



University of Connecticut  
**OpenCommons@UConn**

---

Doctoral Dissertations

University of Connecticut Graduate School

---

12-18-2013

# Mechanisms of Dietary Protein-Induced Changes in Calcium Absorption Efficiency

Jessica D. Bihuniak  
[jessica.bihuniak@gmail.com](mailto:jessica.bihuniak@gmail.com)

Follow this and additional works at: <https://opencommons.uconn.edu/dissertations>

---

## Recommended Citation

Bihuniak, Jessica D., "Mechanisms of Dietary Protein-Induced Changes in Calcium Absorption Efficiency" (2013). *Doctoral Dissertations*. 308.  
<https://opencommons.uconn.edu/dissertations/308>

## **Mechanisms of Dietary Protein-Induced Changes in Calcium Absorption Efficiency**

Jessica Daus Bihuniak

University of Connecticut, 2014

We undertook a randomized, double-blind, crossover study in 14 healthy women who were required to ingest a 6-day low protein diet supplemented with CaSR-AAA (calcium-sensing receptor-activating amino acids), DAA (dibasic amino acids) or methylcellulose capsules (control). All subjects ingested all three diets in random order. Prior to each experimental diet subjects consumed an 11 day adjustment diet. Calcium absorption was measured on day 5 of each dietary intervention using dual-stable calcium isotopes. The clinical trial tested the hypothesis that dietary protein affects the efficiency of intestinal calcium absorption by activating the calcium sensing receptor (CaSR). Supplementation with CaSR-AAA did not significantly increase Ca absorption. However, a trend towards improved Ca absorption was observed with DAA ( $25.2 \pm 1.4\%$  vs.  $22.3 \pm 1.4\%$ ,  $P=0.094$ ).

To further elucidate the mechanisms by which dietary protein improves Ca absorption, we established and validated a  $^{45}\text{Ca}$  in vitro transport model. Differentiated Caco-2 cells were exposed to different concentrations of amino acids for 6 and 72 hours. Prolonged amino acid treatment significantly enhanced total transepithelial Ca transport ( $P=0.0048$ ).

Subsequent in vitro studies utilized our recently validated transport model and focused on the functional role of Cldn-2 in total transepithelial Ca transport. Cldn-2 is a tight junction protein that was previously shown to be upregulated by dietary protein in rats. Total transepithelial Ca transport was significantly reduced when Cldn-2 was stably knocked down in Caco-2 cells ( $P<$

Jessica Daut Bihuniak – University of Connecticut, 2014

0.0001). The contribution of Cldn-2 to amino acid-induced increases in Ca transport will be explored in future studies.

# **Mechanisms of Dietary Protein-Induced Changes in Calcium Absorption Efficiency**

Jessica Dauz Bihuniak

B.S., University of Connecticut, 2007

M.S., University of Connecticut, 2010

A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

at the

University of Connecticut

2014

APPROVAL PAGE

Doctor of Philosophy Dissertation

**Mechanisms of Dietary Protein-Induced Changes in Calcium Absorption Efficiency**

Presented by

Jessica Dauz Bihuniak, MS, RD

Major Advisor \_\_\_\_\_

Jane E. Kerstetter

Associate Advisor \_\_\_\_\_

Karl L. Insogna

Associate Advisor \_\_\_\_\_

Nancy R. Rodriguez

Associate Advisor \_\_\_\_\_

Yih-Woei Fridell,

Associate Advisor \_\_\_\_\_

Anne Kenny

University of Connecticut

2014

## **Acknowledgements**

We are thankful for the support of our funding agencies: the Yale Bone Center, Dairy Research Institute Project Number 1855, and US Department of Agriculture/Agriculture and Food Research Initiative Program 2009-65200-05920. Additional support was provided by the Clinical and Translational Science Award grant number UL1 RR024139 from the National Center for Research Resources and the National Center for Advancing Translational Science, components of the National Institutes of Health (NIH), and NIH roadmap for Medical Research.

The research summarized in this dissertation is one of collaborative efforts and could not have been accomplished alone. Both the clinical and in vitro studies stemmed from prior work conducted by Dr. Karl Insogna, Dr. Jane Kerstetter and Dr. Erin Gaffney-Stomberg. The clinical trial was made possible by the Metabolic Kitchen of the Yale University Hospital Research Unit, the Mineral Metabolism Laboratory at Yale School of Medicine and the O'Brien Laboratory at Cornell University. The bench research was conducted in Dr. Karl Insogna's lab at Yale through a long-standing collaboration with my major advisor, Dr. Jane Kerstetter.

I would like to especially thank Dr. Jane Kerstetter who advised and supported me through my master's and doctoral research. Not only is she a role model for young female scientists, but an exceptional educator. She has inspired me to pursue a career that combines both teaching and nutrition research. Her sense of humor and love for nutrition has made for a pleasurable graduate school experience. Of the many words of advice Dr. Kerstetter has passed on, the following phrase is one that continues to resonate with me, "If you love what you do, you won't work a day in your life".

I owe many thanks to Dr. Karl Insogna as he has been an outstanding mentor and teacher. His door was always open (beyond normal working hours) when I needed guidance or advice on an experiment. He has created a laboratory that is both challenging and stimulating, yet warm and supportive. I have thoroughly enjoyed working with him and his lab members throughout the past 6+ years. I attribute my current laboratory skill set and enthusiasm for research to him.

Lastly, I would like to thank my family. My parents, Sharon and Steve, instilled in me the importance of education and work ethic from a young age. They are two exceptional role models who have provided continued love and support. My sister, Alex, has lent an ear to listen, been there for me when I was discouraged, and provided support in more ways than one. My in-laws, Ruth and Pete, have encouraged me throughout my graduate work and have been there to celebrate each milestone. But, if not for my husband Pete, I would not have been as successful in my accomplishments. He was patient when I was overwhelmed and stressed, excited when I was at the cusp of a new discovery, understanding when I was occupied with work and a proud companion when the journey came to an end.

## Table of Contents

<b>Chapter 1: Introduction.....</b>	<b>1</b>
<b>Chapter 2: Macronutrient regulation of calcium transport: a mini-review.....</b>	<b>3</b>
<b>Chapter 3: Supplementing a low protein diet with dibasic amino acids results in higher urinary calcium excretion in young women.....</b>	<b>12</b>
<b>Chapter 4: Establishment of an in vitro transport model for evaluating the effects of amino acids and claudin-2 on total transepithelial Ca transport.....</b>	<b>31</b>
<b>Chapter 5: Conclusions and future study directions.....</b>	<b>52</b>
<b>References.....</b>	<b>54</b>



## **Chapter 1:**

### **Introduction**

There is an increasing interest in finding dietary alternatives for both prevention and treatment of bone loss as osteoporosis continues to afflict an estimated 10 million people in the United States. Environmental factors such as nutrition play an important role in causing and preventing osteoporosis. In particular, dietary protein is an important nutrient that affects the way our bodies handle Ca. The primary focus of this dissertation was to begin to elucidate the mechanisms by which dietary protein improves Ca absorption efficiency by pursuing three specific aims: 1. determining which functional group of amino acids is most responsible for the effects of dietary protein on intestinal calcium absorption, 2. developing an in vitro transport system to assess the direct effect of amino acid on enterocyte Ca transport, 3. evaluating the functional role of tight-junction protein Claudin-2 (Cldn-2, a gene hypothesized to be involved in dietary protein's effects on Ca absorption) in total transepithelial Ca transport.

Chapter 2 reviews macronutrient regulation of intestinal Ca handling. The chapter summarizes epidemiological, clinical, animal and in vitro research that explores the effects of carbohydrates, fat and/or protein on Ca absorption and transport.

Chapter 3 is currently in the first review for publication in The Journal of Nutrition. This chapter is dedicated to specific aim 1, that is to determine which functional group of amino acids is most responsible for the effects of dietary protein on intestinal calcium absorption. This human intervention trial sought to recapitulate the effect of increasing dietary protein on Ca absorption by adding selected groups of amino acids to a low protein diet (0.7 g/kg/d) such that their total dietary content is equal to that of our previously published high-normal protein diet (2.1 g/kg/d). Supplementing a low protein diet with dibasic amino acids (arginine and lysine) resulted in a trend towards increased Ca absorption.

Chapter 4 contains in vitro experiments designed to pursue specific aim 2 and 3. We developed a  $^{45}\text{Ca}$  transport model in Caco-2 cells to assess the direct effect of amino acids on enterocyte Ca transport. Our preliminary results demonstrate significant increases in total transepithelial Ca transport with prolonged, but not acute, amino acid treatments. Alternatively, we utilized our in vitro model to evaluate the role of Cldn-2 in total transepithelial Ca transport. Previously, Cldn-2 was identified as a tight-junction protein whose expression is increased in rats consuming a high protein diet. This novel finding fostered further investigation of this gene in Ca transport. Stable suppression of Cldn-2 resulted in a significant reduction in total transepithelial Ca transport. Future studies will evaluate the functional role of Cldn-2 in amino-acid induced increases in Ca absorption.

Chapter 5 summarizes finding from the in vivo and in vitro studies presented in Chapters 3 and 4. As a follow up to the completed experiments, potential studies addressing current research gaps are proposed in this chapter.

## Chapter 2:

### Macronutrient regulation of calcium transport: a mini-review

#### Introduction

Dietary Ca is absorbed by both transcellular and paracellular mechanisms. When Ca intake is low, the ATP-dependent transcellular calcium transport pathway predominates in the upper duodenum. The three step process involves: apical Ca entry via Transient Receptor Potential cation channel, subfamily V, member 6 (TRPV 6) and to a lesser extent, by TRPV 5, facilitated diffusion of Ca by calbindin- $D_{9k}$  and basolateral extrusion via plasma membrane Ca ATPase (PMCA1b) or the Na<sup>+</sup>/Ca<sup>2+</sup>-exchanger (NCX1) (1). Down regulation of the transcellular Ca transport pathway results from a normal or high dietary Ca intake, and in turn, paracellular Ca absorption increases throughout the small intestine (2). Passive Ca absorption has previously been defined as ion selective diffusion through the tight-junction of epithelial cells. However, increasing interest in the area of heritable disease states has drawn attention to the protein composition of the tight junction (1). The tight junction is comprised of transmembrane proteins, cytoplasmic scaffolding proteins and cytoskeletal proteins that create a dynamic and regulated environment. Particularly, transmembrane proteins, claudins (Clns) and occludin, have a functional role in selective ion permeability (1, 3, 4). For instance, Cldn-2 forms channels that increase tight junction permeability of water and sodium (5, 6).

Vitamin D is the most well-established nutritional regulator of Ca absorption (2). 1,25-dihydroxyvitamin D mediates active Ca transport by increasing expression of brush-border membrane Ca channels, calbindin- $D_{9k}$  and the basolateral extrusion system (1). 1,25-dihydroxyvitamin D increases the expression of two Ca-selective tight junction pore formers, Cldn-2 and -12, which suggests a novel regulatory function of vitamin D in paracellular Ca transport (7). Alternatively, macronutrient regulation of Ca absorption has been an area of

much debate for over half a century. This mini-review summarizes past and emerging research demonstrating modulation of the Ca absorption pathway, with a primary emphasis on the paracellular transport route, by dietary carbohydrates, fat and protein, and calls attention to areas requiring further investigation.

### **Effect of select carbohydrates on calcium absorption**

Since the 1940's, milk has gained the attention of nutritionists for its rich Ca content and impact on bone health (8). Therefore, it is not surprising that other constituents of milk have been evaluated for their potential Ca absorption enhancing properties. The lactic acid byproduct from the metabolism of "milk sugar" or lactose by lactobacilli was previously hypothesized to lower pH content of the gastrointestinal tract, providing a mechanism by which lactose increases Ca absorption (9). The acidic pH hypothesis was briefly replaced with the theory that certain mono-, di- and oligosaccharides are osteogenic stimulators (10). However, research demonstrating the rapid acting effects of lactose on Ca absorption challenged the bone cell theory, and the potential Ca absorption/retention enhancing properties of lactose were revisited. Subsequent studies in rats demonstrated an increase in femur  $^{85}\text{Sr}$  uptake (considered an index of Ca absorption) following co-administration of Ca and lactose in a segment of the ileum compared to Ca alone, or Ca and lactose in adjacent segments (11). An acute oral dose of lactose enhanced Ca absorption above that seen with the control dose, and to a slightly greater extent than lysine treatment (11), which has also been proposed to increase Ca absorption in rats and humans (12, 13). Conversely, two week feeding of glucose or lactose had no effect on  $^{45}\text{Ca}$  femur content (11). However, injecting carbohydrates including lactose into ileal sacs from rats resulted in increased  $^{45}\text{Ca}$  femur content with all administered carbohydrates within 4 hours after treatment. The slow rate of carbohydrate absorption was proposed as the mechanism by which carbohydrates exert an effect on Ca absorption, thus enabling them to reach the lower segment of the small intestine. The enhancing effect was not observed in the duodenum (9).

Additional research in animal models corroborates these findings (14, 15). These results suggest that carbohydrates including lactose, glucose, sucrose and fructose would more likely exert an effect on the paracellular absorption route rather than the transcellular Ca transport pathway, as the predominate enhancing effect was observed in the ileum and not the duodenum (2). Furthermore, TRPV6, the primary apical transporter involved in transcellular Ca transport, is highly expressed in the duodenum, and present at a much lower extent in the ileum, making it less likely that the aforementioned carbohydrates would impact TRPV6 expression (1).

Schuetz and colleagues undertook a jejunal perfusion study in 16 healthy adults to assess the effect of lactose and the combination of glucose and galactose on calcium absorption (16). Lactose did not significantly alter Ca absorption compared to control or treatment with glucose/galactose. Yet, glucose/galactose, the monosaccharides found in lactose, significantly increased net Ca absorption compared to mannitol (control). Water and sodium permeability paralleled Ca absorption. A solvent-drag effect was proposed as the mechanism by which simple carbohydrates could enhance paracellular Ca absorption. More recently, a cross-over trial in eleven lactose tolerant men and women examined the effect of lactose on fractional Ca absorption using pharmacokinetics of orally administered strontium (17). Subjects ingested a bolus of lactose, glucose and galactose, or control (strontium alone) in random order. Food record analysis revealed consistency in kcals, carbohydrate, fat, protein, calcium and phosphorus intake between the three study groups 4-days prior to each Ca absorption assessment. There were no significant differences in Ca absorption 240 min after ingestion of either carbohydrate bolus or control, suggesting a one-time bolus of lactose is ineffective in enhancing Ca absorption in this population. However, the study had limitations. Compared to the oral strontium bolus employed by Zittermann et al. to evaluate fractional Ca absorption, dual-stable Ca isotopes would have provided a more accurate assessment as they are

considered the preferred methodology (18). Dietary intake prior to each absorption test was not tightly controlled. The relatively small sample size and short duration may have precluded the investigators from capturing a measurable effect. Lastly, the decline in lactase from childhood to adulthood is well-established, with the majority of the world's adult population being effected (19). Thus, it is not unreasonable to question the appropriateness of the selected population.

Population based studies have demonstrated that for a variety of ethnic groups expression of lactase is much higher in infancy than in adulthood (20), suggesting that lactose supplementation in the early stages of life may provide beneficial effects. An acute study in tube fed preterm infants examined the effect of lactose and glucose polymers on Ca absorption (21). Ca absorption was higher in the presence of glucose polymers compared to lactose. The study evaluated Ca absorption 1 hour post feeding and did not assess the impact of chronic carbohydrate ingestion on intestinal Ca handling. In a second study of longer duration, Ca absorption was evaluated by dual-stable Ca isotopes in 18 infants receiving both lactose and lactose-free, maltodextrin based formulas in a randomized, cross-over fashion (22).

Consumption of lactose containing formula for two weeks resulted in significantly higher Ca absorption. Zinc absorption was unaltered by the carbohydrate composition of the formula, suggesting that the effect of lactose may be specific to Ca. Lactose as well as other carbohydrate based formulas may enhance Ca absorption in infancy. However, the overall effect of carbohydrates on Ca economy throughout the lifespan is, if at all, modest and requires further investigation.

### **Effect of certain fatty acids and total fat intake on calcium absorption and transport**

The effect of fatty acids, most notably conjugated linoleic acid (CLA), on calcium transport has been investigated in Caco-2 cells. Jewell and Cashman assessed calcium transport in fully differentiated Caco-2 Bbe cells after acute and chronic exposure to linoleic acid, capric acid, lauric acid, a CLA blend or CLA isomers (23). Total transepithelial <sup>45</sup>Ca transport and estimated

transcellular Ca transport were unaffected by both acute and chronic fatty acid treatments. Compared to the control treatment (vehicle), chronic exposure to capric acid, lauric acid, and trans-10, cis-12 CLA isomer significantly increased fluorescein flux, a non-specific marker of paracellular permeability, and decreased TEER. Conversely, subsequent reports have demonstrated significant increases in total transepithelial, transcellular and paracellular calcium transport in 14-day old Caco-2 cells exposed to CLA isomers (24, 25). Specifically, Jewell et al reported the addition of cis-9, trans-11 or trans-10, cis-12 CLA isomers to regular culture media for 14 days significantly augmented the three assessments of Ca flux compared to vehicle or linoleic acid treatment (24). Murphy et al demonstrated a significant increase in Ca transport after 12 days of trans-10, cis-12 CLA isomer treatment compared to linoleic acid treatment (25). Alternatively, transport was unaltered in cells exposed to the cis-9, trans-11 CLA for 12 days. The inconsistencies in culturing periods between Jewell and Cashman's report and the two later studies may explain their discrepant findings. Although Jewell et al reported no differences in alkaline phosphatase and aminopeptidase N activity in cells cultured for 14 days versus 22 days (24), the activity of brush border membrane enzyme sucrase-isomaltase has been shown to change throughout a 21 day culture period (26). Transport of  $^{45}\text{Ca}$  was 1.8-fold higher in vehicle treated cells cultured for 14 days compared to cells cultured for 22 days (23, 24). Additionally, paracellular transport of fluorescein was approximately 9.4-fold higher in cells exposed to trans-10, cis-12 CLA for 12 days with a culture period of 14 days compared to a 22 day treatment and culture period (23, 25), which provides evidence for differences in monolayer leakiness over various culturing periods. Furthermore, passage number of Caco-2 cells, which was not documented by Jewell and Cashman (23) or Murphy et al (25) can influence the expression of nutritionally relevant transporters and activity of brush border enzymes (27), and may have contributed to the observed difference.

Murphy and colleagues also investigated fatty acid-induced alterations in global gene expression in Caco-2 cells. Expression of calcium transport regulatory proteins, VDR and calbindin  $D_{9k}$ , and tight junction protein, Claudin-4, were found to be upregulated more than 2-fold by trans-10, cis-12 CLA isomer relative to linoleic acid treatment, which is consistent with the observed isomer-specific effects on calcium transport. No effect was seen with cis-9, trans-11 CLA treatment. The authors noted that this is the first study to employ whole genome-wide microarray screen to investigate the effects of different CLA isomers on genes involved in calcium transport in an in vitro model of the intestines. This study provides mechanistic insight into isomer-specific CLA induced increases in calcium transport. The increases in gene expression of VDR and calbindin  $D_{9k}$  by trans-10, cis-12 CLA provides rationale for the observed increase in transcellular calcium transport. However, it is currently unclear as to how the significant induction in Claudin-4 expression directly relates to calcium transport in the intestine. Claudins are a family of transmembrane proteins which have been shown to regulate paracellular transport of ions and water through the tight junctions of epithelia (28). A number of tissues including the intestine and the renal collecting duct have been shown to express Claudin-4 (29, 30). Claudin-4 has been characterized as a seal-forming claudin (31), however, in the presence of Claudin-8 has been shown to form a  $Cl^-$  specific channel in the tight junctions of renal collecting duct cells (32). It is important to note that Murphy et al also demonstrated a significant increase in Claudin-2, a calcium permissive tight junction protein (7), in response to trans-10, cis-12 CLA by microarray screen, but this finding was not confirmed by quantitative RT-PCR (25). More research regarding the effect of CLA isomers on claudin proteins in relation to paracellular calcium permeability is warranted.

The association between dietary fat intake and Ca absorption was recently examined in a retrospective analysis of dual-stable isotopic studies in postmenopausal women (33). Dietary fat intake was positively associated with true fractional Ca absorption. The strongest association



for fat intake and Ca absorption was seen in overweight women compared to obese and lean women. Stepwise multiple regression analysis revealed that dietary fat intake was the second most significant predictor of Ca absorption in all women. Neither carbohydrate nor protein intake were independent predictors of Ca absorption in this model. However, predictors of Ca absorption differed for premenopausal women, as fat intake was not considered an independent predictor of Ca absorption for this age group. The data suggests that dietary fat intake may have Ca absorption enhancing properties in older, but not younger women. Similarly, a stepwise analysis of three dual-stable Ca isotope studies in 50 post-menopausal women found fat intake to be a significant predictor of fractional Ca absorption (34). However, intervention trials assessing the impact of dietary fat on Ca absorption at different stages of life are required to better understand the interplay between dietary fat, age and Ca absorption in women.

### **Effect of dietary protein and select constituents on calcium absorption**

Controversy continues to surround the effect of dietary protein on intestinal Ca absorption. Hunt et al (35) and Ceglia et al (36), reported no differences in calcium absorption between different levels of dietary protein when calcium intakes met or surpassed the RDA (35, 36) and dietary phosphorous was not fixed (36). Higher calcium and phosphorus intakes could have masked any potential regulatory actions of dietary protein on calcium absorption. Conversely, studies using dual stable Ca isotopes report that in the short term, dietary protein profoundly affects intestinal Ca absorption. In particular, Kerstetter et al demonstrated that as compared to a high protein diet (2.1 g protein/kg/d), restricting dietary protein to 0.7 g protein/kg/d results in hypocalciuria caused by a reduction in fractional intestinal Ca absorption (37). Additionally, increasing dietary protein from 1.0 to 2.1 g protein/kg/d resulted in an increase in urinary calcium that is not accompanied by evidence for increased bone resorption. The incremental change in urinary calcium can be nearly quantitatively explained by the improvement in intestinal calcium absorption that accompanies the increase in dietary protein (38). In this

experiment natural food stuffs were used and the dietary content of calcium, sodium and phosphorus were controlled so that they were the same or very similar on both the 1.0 g/kg and 2.1 g/kg protein diets. Dietary protein was increased using both animal and vegetable protein sources.

The constituents of dietary protein responsible for the previously observed increases in Ca absorption are unknown. In particular, caseinphosphopeptides (CPPS) which are a component of milk as well as a digestion product of casein have been hypothesized to increase Ca absorption. Ca binding to CPPS maintains Ca solubility and increases the bioavailability of Ca in the small intestine (39). However, data in animal models have provided inconsistent results. The effect of CPPS on Ca absorption was previously assessed in normal and rachitic chicks using inverted gut sacs and ligated duodenal loop methodology (40). Ca absorption was increased by CPPs in the duodenum and ileum in both chick models. Phosphate, which may be released during the absorption of CPPS, had no effect on Ca absorption. These data suggest that the effect of CPPS on Ca absorption may be independent of vitamin D and phosphate. In ovariectomized rats the effect of CPPS on Ca absorption was dependent on Ca intake (41). Alternatively, an acute study in rats evaluated changes in Ca absorption when whey was replaced with casein and mixed protein sources including CPPS. Type of protein source had no effect on Ca absorption (42). A randomized, double-blind crossover study in postmenopausal women examined the effect of a one-time dose of CPPS-supplemented milk on urinary Ca (UCa) (43). CPPS-supplemented milk did not enhance UCa compared to control milk. The study possessed some major limitations. There was no adjustment period prior to the first dietary intervention and UCa collection. Besides Ca, other nutrients known to affect Ca absorption were not controlled for between study visits and Ca absorption was not directly measured. A more recent crossover, intervention trial assessed the effect of a one-time CPPS bolus on intestinal Ca handling using dual stable Ca isotopes and reported no increase in Ca

absorption with CPPS compared to control (44). Thus, the acute intervention period may not have been of sufficient duration to see an effect.

There are obviously other components of dietary protein that could be responsible for the previously observed increases in Ca absorption (37, 38). In particular, amino acids have been hypothesized to have a direct effect on enterocyte Ca transport. Chapters 3 and 4 summarize the current evidence supporting the role of amino acids in Ca absorption and paracellular Ca transport. In chapter 3 we evaluate the effect of select groups of amino acids on Ca absorption in vivo. Subsequently, the potential mechanism by which amino acids exert an effect on the enterocyte is explored in Chapter 4.

## **Conclusion**

Macronutrient regulation of Ca absorption continues to be an area of much debate. Past and current evidence concerning these nutrients is suggestive of macronutrient-induced alterations in paracellular Ca transport, rather than transcellular pathway activation. It may be that macronutrient regulation occurs at different critical stages throughout the lifespan, such as infancy, childbearing years and post-menopause, with each macronutrient exerting an effect at different periods. It would be of interest to examine the impact of macronutrients on intestinal Ca handling in different age groups and sexes in longer-term clinical trials, as the response is likely to vary between the different groups.

## **Chapter 3:**

### **Supplementing a low protein diet with dibasic amino acids results in higher urinary calcium excretion in young women**

#### **Introduction**

For nearly 90 years, we have known that dietary protein affects calcium metabolism (45). In a meta-analysis of 26 clinical intervention trials in adults where dietary protein was manipulated and urinary calcium was measured, there was a strong linear correlation of the two (46) such that on average, for every 40 g increment in dietary protein, urinary calcium increases by 50 mg. It was widely assumed that the skeleton was the principal source of the additional calcium. Higher protein diets, particularly those including animal sources, generate a fixed metabolic acid load due to the metabolism of sulfur-containing amino acids. The diet-induced acid load is believed to be incompletely accommodated by renal mechanisms, therefore, requiring release of buffer from bone. The liberation of alkali from bone requires osteoclast-mediated bone resorption, which could over a prolonged period of time reduce skeletal mass (47-50). In support of this hypothesis, balance studies in the 1970's reported no change in intestinal calcium absorption as dietary protein increased (51-57). However, more recent studies using dual stable calcium isotopes found that in the short term, dietary protein significantly affects intestinal calcium absorption (37, 38). In particular, Kerstetter et al demonstrated that increasing dietary protein from 1.0 to 2.1 g/kg results in an increase in urinary calcium that is not accompanied by evidence for increased bone resorption. The increment in urinary calcium observed by these investigators was nearly quantitatively explained by a parallel improvement in intestinal calcium absorption (38). Recent work using an experimental rat model found a similar effect of dietary protein on intestinal calcium absorption (58). Initial rates of calcium uptake were increased in brush border membrane vesicles (BBMVs) isolated from rats acclimated to a high protein diet (40%) compared to BBMVs from rats consuming a low protein diet (5%), suggesting that protein

augments intestinal Ca absorption at least in part by increasing transcellular Ca uptake. The constituents of protein-containing foods that are responsible for its effect on intestinal calcium absorption remain unclear. One obvious candidate is amino acids.

Amino acids could impact calcium absorption by altering cellular metabolism in enterocytes or act as extracellular agonists via cell surface receptors or acceptors. Regarding the latter possibility, amino acids are known to be allosteric activators of the calcium-sensing receptor (CaSR), which is expressed throughout the gastrointestinal tract (59). Phenylalanine, tryptophan and histidine are the most potent activators of the CaSR (60, 61). Conigrave et al. (60) first reported amino acid-induced activation of the CaSR at physiologic extracellular calcium concentrations in HEK cells engineered to overexpress the receptor. Busque et al. (62) reported that L-phenylalanine and L-tryptophan at concentrations comparable to those seen postprandially can stereoselectively allosterically activate the CaSR on gastric parietal cells both in vivo and ex vivo. Work by Dawson-Hughes et al. (63) provides further evidence in support of the hypothesis that the CaSR could mediate dietary protein-induced increases in intestinal calcium absorption. They demonstrated that supplementing a low protein diet with phenylalanine and histidine increased urinary calcium; although they did not directly measure intestinal calcium absorption. Another group of amino acids that appears to have effects on intestinal calcium absorption are the dibasic amino acids. In particular, dietary lysine supplementation was reported to increase calcium absorption in individuals with osteoporosis (13). The mechanism by which lysine supplementation induces changes in calcium absorption efficiency is unclear and is likely independent of CaSR activation because lysine is minimally effective as a CaSR agonist (60).

Despite these data, whether specific amino acids or dietary protein as a whole is responsible for the protein-induced increases in calcium absorption remains unresolved. The current human intervention trial was designed to determine if supplementing a low protein diet (0.7 g/kg) with

the CaSR activating amino acids (CaSR-AAA), tryptophan, phenylalanine and histidine, or the dibasic amino acids (DAA), arginine and lysine, augments intestinal calcium absorption to an extent comparable to that seen when dietary protein is increased from low to high.

## **Methods**

### **Subjects**

Sixty-five women (20-40 years old) with a body mass index (BMI, kg/m<sup>2</sup>) ranging from 18 to 28 were screened for eligibility. Subjects were recruited through flyers and advertisements in the Yale Bulletin between the years 2010 and 2012. Recruitment was limited to non-Hispanic Caucasian or Asian adults because these two ethnic groups are at highest risk for osteoporosis (64). Potential subjects were excluded if they were taking medications known to affect calcium metabolism (anti-osteoporotic medications, glucocorticoids, non-steroidal anti-inflammatory medications, and birth control pills); were pregnant; reported excessive body weight change during the past 6 months; followed intensive physical exercise regimens; smoked; had an eating disorder, food allergies or followed medically prescribed diets; or had renal, gastrointestinal, or bone disease; or amenorrhea. Subjects were asked to stop taking all multivitamin or mineral supplements during the entire study. Throughout the study, subjects continued their usual activities at home, school, or work except on days 5 and 6 of the three experimental periods, at which time they were admitted to the Hospital Research Unit (HRU) of the Yale Center for Clinical Investigation for measurement of calcium absorption. The study was approved by human investigation committees at Yale University and the University of Connecticut. Informed consent was obtained from each participant.

### **Experimental Design**

**Diets:** The study protocol included 3 cycles, each consisting of an 11 day adjustment diet followed by 6 days of an experimental diet. The three experimental low-protein diets (control,

CaSR-AAA supplementation, DAA supplementation) were provided in random order. The experimental and adjustment diets are similar to those described in previous reports (37, 38, 65). The Yale Center for Clinical Investigation Research Dietitian and her staff prepared all meals for every study subject during the feeding portion of the study (last 3 days of the adjustment diet and 6 days of the experimental diet). For the first 8 days of each adjustment period, subjects were instructed by the research dietitian to self-select their diets to contain a dietary protein intake of 1.2 g/kg, 2300 mg (100 mmol) of sodium and 800 mg (20 mmol) of calcium. The low-protein experimental diet (0.7 g/kg) consisted of variety of foods common to the western diet and contained 800 mg (20 mmol) calcium, 2300 mg (100 mmol) sodium, 800-1,200 mg (26–39 mmol) phosphorus and 13 grams of fiber. For each study subject, food items remained the same on all three experimental diets. Each subject began with an energy intake of 30-36 kcal/kg (125–150 kJ/kg) and which was adjusted in 200-300 kcals/kg (840–1260 kJ/kg) increments (with simple sugars and fats) during the experimental period to maintain body weight within 1% of initial weight. The macronutrient and mineral composition of the experimental diets was calculated from the U.S. Department of Agriculture Handbook No. 8 and manufacturer's information. The primary sources of calcium in the experimental diets were dairy foods and a chewable calcium carbonate (Tums, GlaxoSmithKline, Pittsburgh, PA). Caffeine-containing beverages were limited to one a day and alcohol was not permitted. Distilled water was provided ad libitum. After the final urine collection on day 6, subjects resumed their usual, unstructured diet, and subsequently participated in the second and third dietary cycles.

**Amino Acid Supplements:** L-amino acids were purchased from Ajinomoto Pharmaceuticals (Ajinomoto Food Ingredients LLC, Chicago IL, 60631) and dispensed in capsules by the Yale Investigational Drug Pharmacy. The total milligrams of supplemental amino acids for a given day were divided among the three meals in amounts proportional to the total protein in each meal. The amount of each amino acid added to the low-protein diet was the amount contained

in the 1.4 g/kg increment in dietary protein required to increase protein intake from low (0.7 g/kg) to high (2.1 g/kg) (i.e.  $2.1 - 0.7 = 1.4$ ). Depending on a subject's body weight, this required between 5 and 7 capsules with each meal.

The average amino acid content (mg) per gram of total protein in the low and high protein diets (shown in **Table 1**) was used as the basis for determining amino acid supplementation (37, 38).

A 60 kg female is used in the following example illustrating how amino acid supplementation was calculated for the study. The baseline low protein diet for a 60 kg female subject would contain 42 grams of protein ( $0.7 \text{ g/kg} \times 60 \text{ kg}$ ) consumed over 3 meals. The subject would require 126 grams of protein ( $2.1 \text{ g/kg} \times 60 \text{ kg}$ ) on the high protein study diet. The increment in dietary protein required to increase her protein intake from 0.7 g/kg to 2.1 g/kg is 84 grams. This subject's three experimental diets will be:

1. 42 g of protein plus placebo (methylcellulose).
2. 42 g of protein plus 2268 mg of histidine ( $27^* \times 84$ ), 6384 mg of phenylalanine ( $76^* \times 84$ ) and 924 mg of tryptophan ( $11^* \times 84$ ).
3. 42 g of protein plus 4368 mg of arginine ( $52^* \times 84$ ) and 5628 mg of lysine ( $67^* \times 84$ ).

\* These values were obtained by averaging the amino acid content of the low and high protein diets previously utilized by Kerstetter et al (37, 38) and represent the mg of each amino acid per gram of dietary protein (last column of Table 1).

**Biochemical Sample Collection:** Between Days 0 and 1 and Days 4 and 5, subjects collected 24-hr timed urine excretions for measurement of calcium, phosphorous, and sodium. On Day 1 and 5 of the experimental diet fasting blood was drawn for measurements of serum 1,25-dihydroxyvitamin D, parathyroid hormone (PTH) and creatinine. Glomerular filtration rate (GFR) was estimated from serum creatinine, age, sex, and race using the National Kidney Foundation on-line calculator MDRD study equation. Serum levels of 25-hydroxyvitamin D were determined



and corrected (if needed) in each study subject prior to beginning each adjustment diet to assure a vitamin D-sufficient status. If the serum 25-hydroxyvitamin D was less than 67 nmol/L but greater than 50 nmol/L subjects received a one-time correction dose of 50,000 IU of vitamin D<sub>2</sub>. Subjects with a serum 25-hydroxyvitamin D level less than 50 nmol/L were given two correction doses of 50,000 IU of vitamin D<sub>2</sub> separated by five days. Serum 25-hydroxyvitamin D was retested at least two weeks after supplementation. Subjects were not permitted to start their adjustment diet until their serum 25-hydroxyvitamin D was at least 67 nmol/L.

**Measurement of Intestinal Calcium Absorption:** Intestinal calcium absorption was measured using dual stable calcium isotopes as previously described (38). In brief, 0.125 mg of <sup>44</sup>Ca/kg body weight was administered orally in 3 divided doses with each meal in proportion to the calcium content of the meal. The isotope was equilibrated in milk for 18-24 hours prior to administration. Immediately after the breakfast meal on day 5, 0.02 mg of <sup>42</sup>Ca/kg (for experimental diets 1 and 3) or 0.004 mg/kg of <sup>43</sup>Ca (for experimental diet 2) were administered intravenously, after which urine was collected for the next 34 hours in three urine pools (8h, 12h, 14h). The intravenous isotope was changed for every other experimental diet to ensure that there was no carryover of isotope from one diet to the next.

Calcium isotope ratios were measured using a Thermo Scientific Triton TI magnetic sector thermal ionization mass spectrometer (TIMS; Thermo Fisher Scientific Inc, Bremen, Germany). A ratio was made between each administered calcium tracer (<sup>42</sup>Ca, <sup>43</sup>Ca and <sup>44</sup>Ca) and another naturally occurring calcium isotope (i.e.: <sup>48</sup>Ca). Fractional calcium absorption was determined as the ratio of the cumulative oral tracer recovery to the cumulative IV tracer recovery in the 34-h urine collection obtained post-dosing. True calcium absorption was calculated as the product of fractional calcium absorption and the calculated calcium intake (38).

**Assays:** Twenty four hour urinary calcium, urinary phosphorous and serum creatinine were measured on an AlfaWasserman ACE® analyzer (Alfa Wassermann Diagnostic Technologies, LLC, West Caldwell, NJ). Urinary sodium was measured in the Clinical Chemistry Laboratory of the Yale–New Haven Hospital. Intact PTH was measured by radioimmunoassay (Total Intact PTH Assay, Scantibodies Laboratory Inc, Santee, CA). Vitamin D metabolites were measured by radioimmunoassays (Diasorin, Inc Stillwater, MN).

**Statistical Analyses:** The estimated number of subjects required to detect a change in calcium absorption is based on the hypothesis that adding CaSR-AAA to a low-protein diet will increase intestinal calcium absorption to an extent similar to that observed when the subject's dietary protein intake is increased from 0.7 to 2.1 g/kg. Since it is possible that amino acids besides those that activate the CaSR also affect intestinal calcium absorption, we estimated the effect size for the CaSR-AAA to be approximately 70% of that seen with the 2.1 g/kg diet. We reported that increasing dietary protein from 0.7 to 2.1 g/kg increased calcium absorption from  $19 \pm 5.0\%$  to  $26 \pm 8.0\%$  (an increment of  $7 \pm 6.5\%$ ;  $P < 0.01$ ) (37). As just noted, to be conservative we estimated an effect size approximately 30% less than that (a  $5 \pm 6.5\%$  increase in calcium absorption). Using this effect size and standard deviation, a sample size of 14 provides a power of 0.80 with  $P = 0.05$  (calculated using GraphPad StatMate® version 2.0a for Macintosh; GraphPad Software, San Diego, CA).

Analyses were performed using SPSS version 12.0 for Windows (SPSS Inc, Chicago, IL). The graphical summary was generated using Prism software (version 4.0, 2004, GraphPad Software). All values are presented as means  $\pm$  SEM. The Shapiro–Wilks test was used to test data for normality. All data were normally distributed except for serum 25-hydroxyvitamin D and 24-h urinary calcium at baseline and intact PTH at baseline and day 5 of all three interventions. A repeated measures ANOVA or Friedman test (for non-normally distributed data) was used to assess differences in baseline measures between the three experimental diets. A paired t-test

or Wilcoxon signed ranked test (for non-normally distributed data) was used to assess differences between each amino acid supplementation group and the control diet at day 5 and differences between baseline (day 1) and day 5 of each experimental diet. A comprehensive assessment of the primary outcome variable, intestinal calcium absorption, was performed by calculating mean effect sizes for the two interventions. This was done by the following calculation: mean absorption on the amino acid supplemented diet for the entire study group minus the mean absorption on the control diet for all study subjects divided by the standard deviation of the difference between the control diet and each amino acid supplemented diet (66). A probability level of  $P \leq 0.05$  indicated statistical significance and  $P > 0.05$  but  $< 0.10$  indicated a trend.

## Results

**Figure 1** summarizes study subject recruitment, enrollment and randomization. Of the 65 subjects screened for eligibility, 51 subjects did not meet the inclusion criteria or were not interested in participating after receiving a detailed explanation of the study. Fourteen healthy premenopausal women with a mean age of  $27.8 \pm 1.2$  and a BMI of  $23.7 \pm 0.9$  were enrolled and randomized.

### Serum and Urine Metabolites

Serum 25-hydroxyvitamin D was within normal limits at the start of each subject's adjustment diet and was not significantly different between the three dietary interventions (control:  $88.1 \pm 4.8$  nmol/L, CaSR-AAA:  $88.1 \pm 4.6$  nmol/L, DAA:  $87.5 \pm 4.3$  nmol/L,  $P=0.67$ ) (67). Baseline measures of serum and 24-h urine metabolites did not differ significantly between the three diets (**Table 2**). Changes observed in calcium-related metabolites during the three experimental diets are presented in Table 2. No significant differences were observed in 1,25-dihydroxyvitamin D or intact PTH between baseline and day 5 of each intervention. Analyses of

the day 5 values for PTH and 1,25-dihydroxyvitamin D revealed no significant differences among the three interventions. Urinary sodium and GFR remained stable with no significant differences observed at any time point for the duration of the study. As expected, GFR was above 60 mL/min/1.73 m<sup>2</sup> in all subjects throughout the entire study period. At day 5 urinary calcium was lower than the value at baseline in all three experimental diets, however, this change only reached statistical significance during the control diet (control: 37.4% mean decrease,  $Z=-2.67$ ,  $P=0.008$ ; CaSR-AAA:  $Z=-0.91$ ,  $P=0.36$ ; DAA:  $Z=-1.48$ ,  $P=0.14$ ). At day 5, urinary calcium was significantly greater with the DAA supplementation than the control diet ( $P=0.039$ ) (Table 2, **Figure 2A**). Twenty four-hour urinary phosphorus on day 5 of the three study diets was found to be significantly lower than at baseline (control:  $P=0.001$ , CaSR-AAA:  $P=0.047$ , DAA:  $P=0.008$ ).

### **Calcium Absorption**

As shown in Table 2, there was no significant difference in calcium absorption between the diet supplemented with CaSR-AAA and the control diet at day 5 (effect size =  $0.12 \pm 0.09$ ,  $P=0.64$ ). However, there was a non-significant trend toward increased calcium absorption with DAA supplementation as compared to the control diet ( $P=0.094$ ), with 10 of the 14 subjects evidencing higher absorption. Because dietary calcium was fixed at 20 mmol/d, intestinal calcium absorption on the low protein diet supplemented with DAA was  $0.6 \pm 0.3$  mmol/d higher than on the control diet. Calculation of effect size revealed that this observed difference in mean intestinal calcium absorption is considered to be a medium effect ( $0.54 \pm 0.1$ ) (68). As can be seen in Figure 2, the increase in intestinal calcium absorption mirrored the change in urinary calcium observed during the two diets. The mean difference in urinary calcium at day 5 between the control and DAA supplemented diets was  $0.9 \pm 0.4$  mmol/d.

## Discussion

We employed dual stable calcium isotopes to evaluate the effect of a low-protein diet supplemented with either DAA or CaSR-AAA on intestinal calcium absorption. The intestinal handling of calcium was not influenced by the addition of CaSR-AAA to a low protein diet. Short-term supplementation with DAA resulted in a significant increase in urinary calcium and augmented calcium absorption without significant changes in PTH or 1,25-dihydroxyvitamin D during the three diet interventions, suggesting that the change in calcium excretion is due to improved intestinal calcium absorption and not an induced renal calcium leak.

It is traditionally assumed that the dietary protein-induced increases in urinary calcium results from the release of skeletal buffer and calcium in response to the metabolic load imposed by sulfur-containing amino acids (47-50). Our data provide evidence for amino acid-induced increases in urinary calcium independent of changes in dietary sulfur content, since arginine and lysine do not contain sulfur. Therefore, the observed increase in urinary calcium is very likely attributable to the rise in intestinal calcium absorption, rather than an increase in skeletal catabolism.

Our laboratory has previously reported increased intestinal calcium absorption during a high-normal protein diet (2.1 g/kg), consisting of both animal and vegetable protein, when compared to both low (0.7g/kg) and medium (1.0 g/kg) protein intakes (37, 38). In these prior studies dual stable calcium isotopes were also used to assess calcium absorption, calcium was fixed at 19.8-20 mmol/d and nutrients known to affect calcium metabolism were tightly controlled. Under these study conditions, when dietary protein was increased from 0.7 to 2.1 g/kg, intestinal calcium absorption rose by 36.8% (37). The current study supplemented the same low protein diet with the amount of the amino acids contained in the 1.4 g/kg increment in dietary protein that was required to increase protein intake from 0.7 to 2.1 g/kg. Intestinal calcium absorption

was 13% higher with dibasic amino acid supplementation compared to the low-protein control diet. The change in calcium absorption in the present study reflects roughly a third of the change in absorption observed when dietary protein was increased by 1.4 g/kg using mixed food sources. Thus, the addition of DAA to the low protein diet did not increase intestinal calcium absorption to the same extent as when dietary protein sources were manipulated. However, DAA supplementation resulted in a quantifiable change in intestinal calcium absorption which can explain 67% of the 0.9 mmol change in urinary calcium. Since intestinal calcium absorption declines with aging and after menopause even a modest improvement in intestinal calcium absorption could have long-term physiological significance. Thus, most estrogen-deficient postmenopausal women are in negative calcium balance and an intervention that improves calcium homeostasis could potentially protect against long-term deleterious effects on skeletal integrity.

Research in animal models supports an effect of DAA on calcium economy (12, 69). Over 50 years ago, Wasserman et al. (69) measured  $^{45}\text{Ca}$  in the femur of young male rats after the enteral administration of an individual amino acid bolus. Of the ten essential and eight non-essential amino acids studied, L-lysine followed by L-arginine resulted in the greatest accumulation of  $^{45}\text{Ca}$  in bone. Compared to the control animals, administration of L-lysine and L-arginine caused 1.7-fold and 1.6-fold increases in  $^{45}\text{Ca}$  accretion in bone, respectively. In subsequent work, these investigators evaluated the interaction between lysine and other nutrients known to effect calcium metabolism in vitamin D deficient rats (12). Treatment with L-lysine or vitamin D enhanced calcium absorption above that seen with control treatment, but not to the same extent as when they were provided in combination. The authors hypothesized that L-lysine and vitamin D may be acting via different cellular pathways to increase intestinal calcium absorption. Taken together, the studies by Wasserman et al and the present study support the notion that additional studies to clarify the role of DAA in calcium metabolism are

warranted. At present, it is unclear if DAA exert an effect on the transcellular, paracellular or both calcium transport mechanisms.

The current study also evaluated a second group of amino acids (phenylalanine, tryptophan and histidine) because they allosterically activate the CaSR. We found no effect of CaSR-AAA on calcium absorption. In contrast, we found an increase in urinary calcium from the DAA supplementation in comparison to the control. It is unlikely that the slight effect we observed of DAA on intestinal calcium absorption is mediated by the CaSR. Conigrave et al. (60) demonstrated that arginine and lysine were not very effective in activating the CaSR in HEK-293 cells stably expressing the receptor.

Dawson-Hughes et al. (63) examined the effects of selectively supplementing a low protein diet with either CaSR-AAA, phenylalanine and histidine, or the branched chain amino acids (BCAA), isoleucine and leucine, on urinary calcium. There was no significant difference in urinary calcium between baseline and day 11 of amino acids supplementation for either study group or between the two study groups at the end of the supplementation period. These findings are consistent with the results of the present study where we found no significant change in urinary calcium with CaSR-AAA supplementation. In a subsequent analysis, mean change in 24-hr urine calcium was also compared between the two groups (63). The addition of CaSR-AAA to a baseline low protein intake of 0.5 g/kg resulted in an  $11 \pm 9$  mg increase in calcium excretion, which was significantly different from the  $20 \pm 9$  mg decline in urinary calcium observed with BCAA supplementation. In contrast to our study, urinary calcium did not change from baseline during the two amino acid interventions. The dietary intervention used by Dawson-Hughes et al differed from the current study in that it contained 0.2 g/kg less dietary protein, 16.1 mg/kg more histidine and 52.5 mg/kg less phenylalanine. Differences in study design, study duration and amino acid supplementation may have contributed to the discrepancy in directional change in urinary calcium between the present study and the report by Dawson-Hughes et al.

A BCAA intervention was not included in the present study. Two human clinical trials found no effect of BCAA supplementation on urinary calcium or calcium absorption (13, 63). Civitelli et al supplemented osteoporotic patients with three different amino acids including the BCAA, L-valine, which did not induce a rise in intestinal calcium absorption (13). Similarly, Dawson-Hughes et al. (63) reported a blunted calciuric response in subjects consuming a diet rich in leucine and isoleucine compared to those with high CaSR-AAA intakes. There are obviously numerous other combinations of amino acids that need to be explored in relation to calcium homeostasis, which is beyond the scope of this study.

Not all investigators have observed an effect of dietary protein on calcium absorption. Hunt et al (35) and Ceglia et al (36), reported no differences in calcium absorption between different levels of dietary protein when calcium intakes met or surpassed the RDA (35, 36) and dietary phosphorous was not fixed (36). Higher calcium and phosphorus intakes could have masked any potential regulatory actions of dietary protein on calcium absorption. It would be of interest to examine the effect of graded calcium intakes in conjunction with different quantities or combinations of amino acid supplements on calcium economy to determine if this has a protective effect on calcium homeostasis in low dietary calcium environments.

The current study had a number of strengths including a tightly controlled cross-over design with all meals provided by the metabolic kitchen at the Yale Center for Clinical Investigation. Good dietary compliance was evident by consistency in urinary sodium. We targeted young Asian and Caucasian women as these ethnic groups are at highest risk for osteoporosis in later adult life (64). A recent analysis of NHANES (2003-2006) reported that women between the ages of 19-40 who do not use calcium supplements are not meeting the current recommendations for calcium (70). Thus, young Asian and Caucasian women would likely benefit from a nutrient based, low-risk, therapeutic option, such as dietary protein/amino acid supplementation, to maximize calcium absorption. Moreover, protein intake has decreased in women from 1970's to



2006 (71). We chose to study a group of individuals who, as they age, are at greatest risk for osteoporosis and depending on dietary protein intake could benefit from protein/amino acid supplementation to optimize intestinal calcium absorption.

The study also had some limitations. Our sample size was small. It may be that larger studies of longer duration will be needed to further clarify the impact of DAA on calcium economy. Some subjects required vitamin D supplementation prior to starting their adjustment diets. However, the number of study subjects requiring vitamin D supplementation was nearly equal for each dietary intervention (DAA: 7 subjects, CaSR-AAA: 8 subjects, Control: 8 subjects) making unlikely that this confounded our results. Time of menstrual cycle was not controlled for when scheduling each study subject's calcium absorption assessments. In initial studies from our group examining the effect of increasing protein intake on calcium absorption using the same dual-stable calcium isotope methodology as in our current report, we did not control for time of menstrual cycle in premenopausal women (37, 38). We observed a quantitatively comparable effect of dietary protein in men and postmenopausal women in whom cyclical changes in reproductive hormone levels do not occur when compared to premenopausal women. Thus, although we cannot exclude a contribution of variations in estrogen levels to our observed results, given the above, this seems unlikely to have made a major contribution.

In summary, calcium absorption was assessed by dual-stable calcium isotopes in healthy premenopausal women consuming a low-protein diet supplemented with CaSR-AAA or DAA for six days. The addition of CaSR-AAA to a low-protein diet did not result in a significant rise or decline in calcium absorption. Urinary calcium was significantly higher with arginine and lysine supplementation, which could potentially be explained by a change in calcium absorption efficiency.

## FIGURE LEGENDS

**FIGURE 1** Flow diagram displaying recruitment, enrollment and randomization of study subjects. Fourteen healthy young women took part in a cross-over design, feeding study where they received three experimental diets in random order (control; CaSR-AAA, CaSR-activating amino acids; DAA, Dibasic amino acids). n=14 for each experimental diet.

**FIGURE 2** Mean urinary calcium (A) and intestinal calcium absorption (B) for fourteen healthy young women on day 5 of the control and DAA supplemented low-protein diets. n=14 for each experimental diet. Significantly different from the control diet by paired t-test: \* $P < 0.05$ , \*\*Different from control, P value  $> 0.05$  and  $< 0.10$ , suggestive of a trend, \*\*\*Effect size =  $0.54 \pm 0.1$ . DAA, Dibasic amino acids.

**Table 1** Average amino acid content per gram of total protein in the low and high protein study diets<sup>1</sup>

<b>Amino acid</b>	<b>Amino acid functional group</b>	<b>Amino acid content of low protein diet (mg/g dietary protein)</b>	<b>Amino acid content of high protein diet (mg/g dietary protein)</b>	<b>Average amino acid content of the diets<sup>2</sup> (mg/g dietary protein)</b>
His	CaSR-AAA	25.3±0.6	28.2±0.2	<b>27</b>
Phe	CaSR-AAA	75.4±0.8	76.5±0.6	<b>76</b>
Trp	CaSR-AAA	11.3±0.2	11.3±0.0	<b>11</b>
Arg	DAA	46.5±1.2	56.8±0.4	<b>52</b>
Lys	DAA	58.9±1.1	74.9±0.8	<b>67</b>

<sup>1</sup> Values are presented as means ± SEM.

<sup>2</sup> Average amino-acid content of the low and high protein diets was used to calculate amino acid supplementation.  
CaSR-AAA, CaSR-activating amino acids; DAA, Dibasic amino acids.

**Table 2** Effect of CaSR-AAA and DAA supplementation on calcium-related metabolites and calcium absorption in young women<sup>1</sup>

	Control		CaSR-AAA Supplementation		DAA Supplementation	
	Day 1	Day 5	Day 1	Day 5	Day 1	Day 5
Subjects, <i>n</i>	14	14	14	14	14	14
Serum metabolites						
1,25-Dihydroxyvitamin D (pmol/L)	151 ± 13	163 ± 8	162 ± 12	155 ± 12	172 ± 14	166 ± 13
Intact PTH (nmol/L)	36 ± 6	31 ± 6	31 ± 7	30 ± 6	32 ± 9	31 ± 7
GFR (mL/min/1.73 m <sup>2</sup> )	91 ± 5	97 ± 6	90 ± 5	91 ± 6	93 ± 5	94 ± 6
24-h urine metabolites						
Sodium (mmol)	90.8 ± 12.6	74.9 ± 6.7	80.9 ± 10.7	86.4 ± 8.2	80.2 ± 7.5	90.1 ± 9.7
Calcium (mmol)	4.6 ± 1.1	2.9 ± 0.3 <sup>a</sup>	3.9 ± 0.6	3.3 ± 0.3	4.2 ± 0.6	3.8 ± 0.5 <sup>b</sup>
Phosphorus (mmol)	18.4 ± 1.8	11.4 ± 1.2 <sup>a</sup>	15.6 ± 1.7	12.0 ± 1.2 <sup>a</sup>	18.2 ± 1.4	12.7 ± 1.3 <sup>a</sup>
Calcium absorption						
Intestinal calcium absorption (%)		22.3 ± 1.4		22.9 ± 2.0		25.2 ± 1.4 <sup>c</sup>

<sup>1</sup>Values are presented as mean ± SEM.<sup>a</sup>Significantly different from baseline of same intervention, *P* < 0.05.<sup>b</sup>Significantly different from control day 5, *P* < 0.05.<sup>c</sup>Different from control day 5, *P* value > 0.05 and < 0.10, suggestive of a trend.

CaSR-AAA, CaSR-activating amino acids; DAA, Dibasic amino acids

**FIGURE 1**

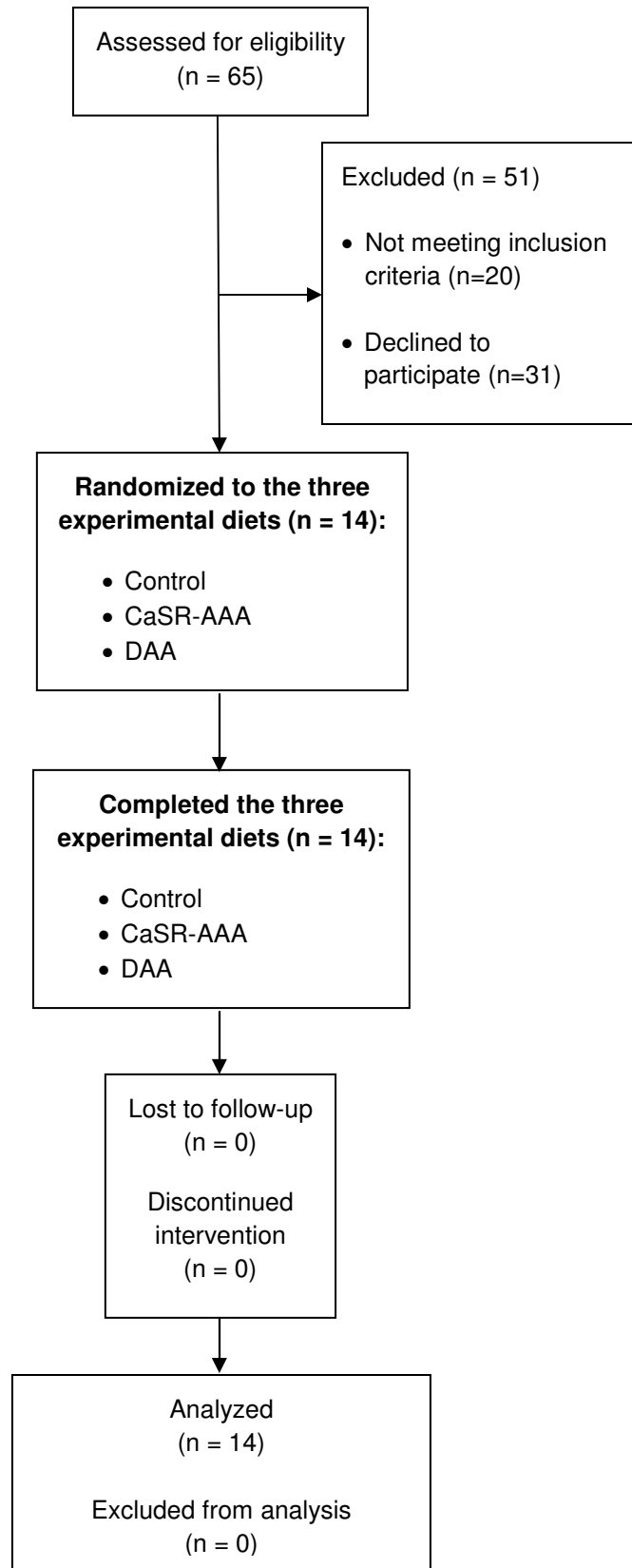
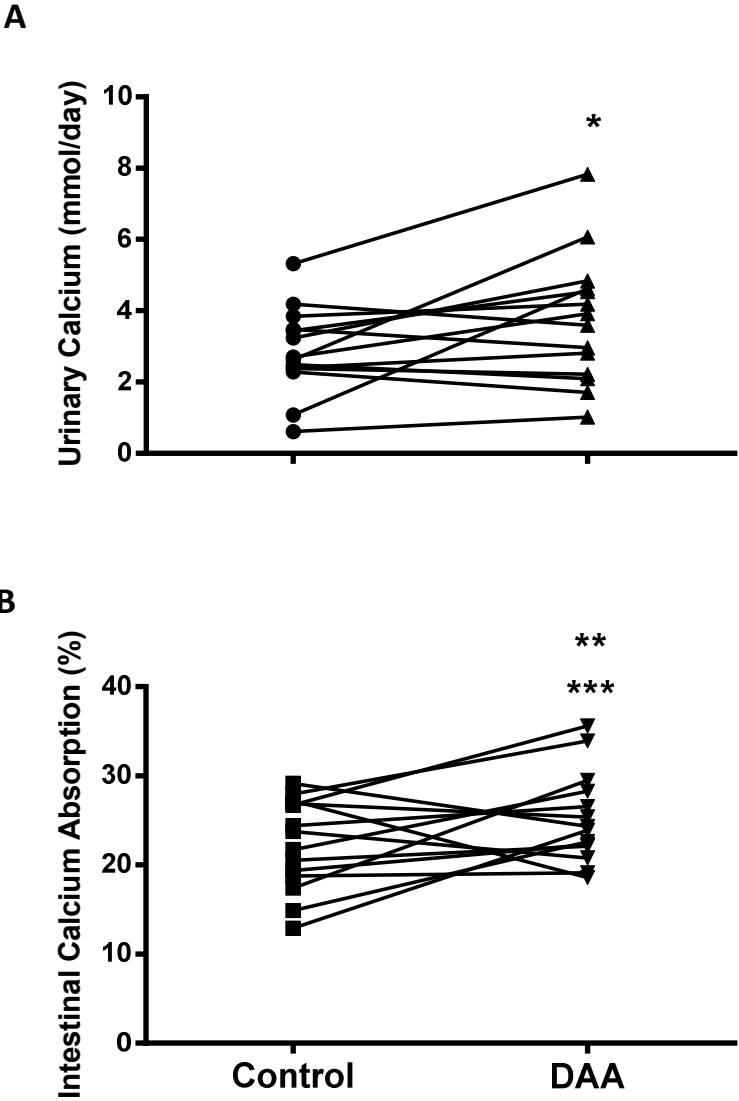


FIGURE 2



## **Chapter 4:**

### **Establishment of an in vitro transport model for evaluating the effects of amino acids and claudin-2 on total transepithelial Ca transport**

#### **Introduction**

Controversy surrounds the role of dietary protein in calcium homeostasis. Dietary protein was thought to have deleterious effects on the skeleton because the endogenous acid load generated from the metabolism of sulfur-containing amino acids might induce osteoclast-mediated bone resorption. The result is Ca being leached from bone as part of a homeostatic mechanism in which bicarbonate is released from mineralized tissue to buffer the acid (47-50). However, Kerstetter et al have demonstrated that moderate increases in dietary protein significantly improve intestinal Ca absorption, in least in the short-term, without increasing bone resorption (38).

The underlying mechanism by which dietary protein increases Ca absorption is unclear. Gaffney-Stomberg et al. established an animal model to begin elucidating the mechanism for protein-induced increases in intestinal calcium absorption (58). Rats were randomly assigned to low (5%) or high (40%) protein diets for 7 days. All diets were isocaloric and contained 0.45% Ca and 0.35% P. In rats, UCa paralleled protein intake. Rats consuming the high protein diet absorbed and retained more Ca compared to the low protein group (48.5% vs. 34.1% and 45.8% vs. 33.7% respectively,  $p < 0.01$ ). Brush border membrane vesicles (BBMV) were prepared from duodena of rats acclimated to the low or high protein diets. Ca uptake was significantly increased in BBMVs from animals on the high protein diet indicating that protein augments intestinal Ca absorption, in part, by increasing transcellular Ca uptake. However, the expression of the known plasma membrane Ca channels, TRPV5 and TRPV6, was not induced by a high protein diet (58). Since expression of TRPV5 and TRPV6 were not altered by dietary

protein, Dr. Insogna's laboratory undertook a whole genome microarray screen using RNA isolated from duodenal enterocytes of rats ingesting the low or high protein diet to look for novel Ca transporters. Claudin-2 (Cldn-2) was found to be upregulated by the high protein diet in the microarray screen, a finding corroborated by qPCR, using duodenal RNA. Cldn-2 is a tight junction protein that exhibits Ca selectivity. Its expression is induced by 1,25-dihydroxyvitamin D. Increased expression of Cldn-2 at the tight junction increases calcium permeability (7). These findings suggest that dietary protein could be affecting calcium transport by a Cldn-2-dependent mechanism.

The constituents of dietary protein responsible for the increases in Ca uptake and absorption are unknown. Furthermore, the function of Cldn-2 in total transepithelial calcium transport is not well established. To investigate the mechanisms by which dietary protein exerts its effects on intestinal Ca transport, and to determine if there is a direct effect of amino acids on enterocyte Ca transport, an in vitro Ca transport model was developed in Caco-2 cells. Since Cldn-2 was upregulated by dietary protein in our rat model, we also sought to determine the contribution of this endogenous protein to Ca transport. Toward this end, Cldn-2 was stably suppressed in Caco-2 cells and Ca transport was evaluated.

## **Materials and Methods**

### *Materials*

Caco-2 Bbe cells were kindly provided by Dr. Mark Mooseker (Dept of Molecular, Cellular & Developmental Biology, Yale University) and purchased from America Tissue Culture Collection (Rockville, MD). High-glucose DMEM, L-glutamine, D-glucose, phenol red powder, HEPES ( $\geq 99.5\%$ ) and D-(+)-Raffinose pentahydrate were purchased by Sigma (St. Louis, MO). Qualified FBS, L-glutamine, non-essential amino acids (NEAA), essential amino acids (EAA), puromycin dihydrochloride and penicillin/streptomycin were purchased from Gibco/Invitrogen (Carlsbad,



CA). KCl, Na<sub>2</sub>HPO<sub>4</sub>, glycine and NaCl were purchased from Fisher Scientific (Suwanee, GA). KH<sub>2</sub>P04 and MgSO<sub>4</sub> were purchased from J. T. Baker (Phillipsburg, NJ). OPTI-FLUOR LSC-cocktail was purchased from Perkin Elmer, Inc (Waltham, MA). Collagen coated polytetrafluoroethylene Transwell permeable supports (Corning Incorporated, Costar, Corning, NY, catalog no. 3491) and Polyester Transwell permeable supports (Corning Incorporated, Costar, Corning, NY, catalog no. 3460) were purchased from Corning Incorporated. pGIPZ Lentiviral Human CLDN2 shRNA was purchased from Fisher Scientific – USA (Pittsburgh PA, catalog # RHS4531-EG9075). Lenti-X HTX Packaging System, Lenti-X Concentrator and Lenti-X GoStix™ were purchased from Clontech (Mountainview, CA). iScript™ cDNA Synthesis Kit and iTaq™ Universal Probes Supermix were purchased from BIO-RAD Laboratories (Hercules, CA). Cldn-2 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) TaqMan® Gene Expression Assays were purchased from Applied Biosystems (Foster City, CA). Protease inhibitors were purchased from Roche Diagnostic (Indianapolis, IN). Mouse anti-Cldn-2 antibody was purchased from Invitrogen (REF325600). Mouse anti-β-actin antibody was purchased from Santa Cruz Biotechnology (sc-47778, Santa Cruz, CA).

### *Cell culture*

Caco-2 cells were grown in T-75 flasks and routinely passaged using Trypsin/0.5% EDTA when 80-90% confluent. Cells were cultured in growth media containing high glucose DMEM, 10% qualified FBS, 1% L-glutamine, 1% non-essential amino acids and 1% penicillin/streptomycin and media was changed every 2-3 days. Caco-2 cells were maintained at 37°C in a 5% CO<sub>2</sub>-95% air atmosphere. Caco-2 cells provide a model of intestinal epithelia, as they exhibit brush border microvilli and apical enzymes including sucrase isomaltase, alkaline phosphatase and aminopeptidase N. For over a decade Caco-2 cells have provided a well-established model for the assessment of small intestinal transcellular flux of a number of compounds that is predictive of in vivo absorption in humans (72, 73). In most of the studies described below, Caco-2 cells

were studied at 21-days post-confluent at which time they are fully differentiated and assume an enterocyte phenotype (74-76).

#### *Amino acid treatments*

As shown in the Table, moderate amino acid (MAA) transport treatment consisted of 20 essential and non-essential amino acids added to transport buffer (TB) and provided 1.914 mg of total amino acids/mL. The final concentration of amino acids provided by the MAA treatment was approximately 1.2-fold higher than the amount found in high glucose DMEM, the primary constituent of regular Caco-2 growth media. High amino acid (HAA) transport treatment had the same amino acid profile as MAA and provided 5.276 mg of total amino acids/mL, which is approximately 3.3-fold higher than the concentration of amino acids in high glucose DMEM (Table). Amino acid pretreatment was comprised of 1% FBS, penicillin/streptomycin and high-glucose DMEM (1% FBS media) supplemented with MAA.

#### *Diffusional phenol red transport studies*

The methodology utilized in the current study for assessing diffusional phenol red flux across Caco-2 monolayers is a modification of that previously published (24, 77). Phenol Red was used to provide a simple assessment of paracellular transport (77). For phenol red transport studies Caco-2 cells were seeded at a density of  $3 \times 10^5$  -  $5 \times 10^5$  cells/well into 6-well plates containing collagen coated polytetrafluoroethylene Transwell permeable supports. For these experiments, Caco-2 cells were studied at 21 days post-confluence. These culture conditions were chosen based on previous work by Suzuki and Hara who examined the effect of raffinose, along with other non-digestible saccharides, on paracellular permeability in Caco-2 cells (78). On the day of an experiment the media was removed and the inserts were rinsed two times with room temperature Dulbecco's phosphate-buffered saline (PBS). The permeable supports containing the cells were transferred to fresh six-well plates containing 2.5 mL of freshly prepared TB ((in mM) 140 NaCl, 5.8 KCl, 1.2 CaCl<sub>2</sub>, 0.34 Na<sub>2</sub>HP0<sub>4</sub>, 0.44 KH<sub>2</sub>P0<sub>4</sub>, 0.8 MgSO<sub>4</sub>, 20

N-Z-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 4 glutamine, and 25 glucose, pH 7.37-7.4) (24, 76, 77). At time zero, 1.5 mL of transport buffer was added to the upper chamber of the Transwells inserts. Upper chamber buffer consisted of the same buffer as the lower chamber except it also contained Phenol Red (500  $\mu$ M). Raffinose (100 mM), a non-digestible oligosaccharide, which non-specifically reduces transepithelial resistance (TER) and increase paracellular permeability in Caco-2 cells, was included in the TB as a positive control (78). Following the addition of transport buffer, cells were incubate at 37°C in a 5% CO<sub>2</sub>-95% air atmosphere (with manual shaking approximately every 30 min for 45 seconds) for 90 minutes, 120 minutes or 4 hours. At the end of each experiment, duplicate 300  $\mu$ l aliquots of lower well buffer was removed and transferred to an eppendorf tube and 30  $\mu$ l of 0.1 N NaOH was added to each sample. Absorbance was read at 560 nm following a 5 min incubation period. The concentration of phenol read appearing in the lower buffer after 90 minutes, 120 minutes and 4 hours of transport was determined using a standard curve of phenol red.

#### *Total transepithelial <sup>45</sup>Ca transport studies*

For total transepithelial <sup>45</sup>Ca transport studies, Caco-2 cells were seeded into 12-well plates with Polyester Transwell permeable supports at a density of 56,000 cells/cm<sup>2</sup>. Similar techniques were utilized to assess the effect of various nutrients on calcium transport (23-25, 76, 77). To validate the in vitro Transwell transport model 1,25-dihydroxyvitamin D, a known regulator of calcium transport (76), was used as a positive control. Cells were pre-treated with 1, 25-dihydroxyvitamin D in 5% FBS growth media for 24 hours prior to the start of the study. 1,25-dihydroxyvitamin D validation experiments mimicked the 4 hour phenol transport studies summarized above, however, <sup>45</sup>Ca was used in place of phenol red to assess total transepithial calcium transport. At Time 0, <sup>45</sup>Ca was added to the upper chamber in the presence of TB (control) or TB to which 1,25-dihydroxyvitamin D (100 nM) had been added. Approximately 5-10 min after addition of <sup>45</sup>Ca triplicate 25  $\mu$ l aliquots were removed from the top (baseline samples)

and bottom wells. After 4 hours, triplicate 50 ul samples were taken from the bottom chambers. Baseline and 4 hour samples were added to scintillation vials containing OPTI-FLUOR LSC-cocktail. Radioactivity was determined using a liquid scintillation counter (Tri-Carb 2800TR). Total transepithelial Ca transport was estimated as the amount of  $^{45}\text{Ca}$  appearing in the basolateral buffer at 4 hours, expressed as a percentage of the total  $^{45}\text{Ca}$  applied to the upper chamber at Time 0. Studies to evaluate the effect of pH on epithelial permeability were conducted using the same methodology, except the duration of  $^{45}\text{Ca}$  transport measurements was extended to 6 hours and the duration of amino acid pretreatment varied as detailed below.

#### *Generation of stable Cldn-2 knockdown in Caco-2 Bbe by lentiviral vector*

Lentiviral preparations were generated according to the manufacturer's instructions. Briefly, 90-95% confluent Lenti-X 293T packaging cells were transfected with pGIPZ Lentiviral vector containing tGFP reporter, puromycin resistance gene, and Cldn-2 specific or non-silencing shRNA (scrambled shRNA, control) using the Lenti-X HTX Packaging System. Forty-eight hours after transfection, lentiviral supernatants were harvested and mixed with the Lenti-X Concentrator. Virus production was verified using Lenti-X GoStix<sup>TM</sup>. Forty-eight hours prior to lentiviral transduction, Caco-2 Bbe cells were plated in 24-well plates at a density of  $3.1 \times 10^4$  cells/well in their complete growth medium. Caco-2 cells were transduced with viral supernatant using Polybrene (as per the manufacturer's protocol), centrifuged at 2,000 RPM for 1.5 hours and incubated overnight. Infected cells were incubated for an additional 48 hours in complete growth medium before the initiation of antibiotic selection with 8  $\mu\text{g}/\text{mL}$  puromycin dihydrochloride (final concentration). Stable transfectants were routinely passaged using Trypsin/0.5% EDTA when 80-90% confluent. Cells were cultured in normal growth media (described above) containing 8  $\mu\text{g}/\text{mL}$  puromycin dihydrochloride with a media change every 2-3 days. To assess infection efficiency, Caco-2 cells were imaged using fluorescence microscopy to visualize GFP expression.

### *Quantitative analysis of Cldn-2 knockdown by Real Time-PCR (qPCR) and Western blot*

#### *qPCR*

Total RNA was isolated from approximately two week post confluent non-infected Caco-2 cells (control), or Caco-2 cells stably transduced with 5 different Cldn-2 specific shRNAs or scrambled shRNA GIPZ lentiviral constructs. cDNA was synthesized using the iScript™ cDNA Synthesis Kit and the RT reaction was performed on a PTC-100 PCR machine (MJ Research Inc., Waltham, MA) using the following protocol: 25°C x 10 min, 43°C x 45 min, 95°C x 5 min. qPCR was performed using a TaqMan® Gene Expression Assay for Cldn-2 (Rn02063575\_s1) and iTaq™ Universal Probes Supermix. GAPDH was used as an endogenous reference (Hs99999905\_m1).

#### *Western Blot*

Caco-2 cells were grown in 60 mm dishes and lysed with RIPA buffer (1% Triton-X 100, 1% Na deoxycholate, 0.1% SDS, 158 mM NaCl, 5 mM EDTA, 10 mM Tris pH 7.2) containing protease inhibitors (Roche Diagnostics Corporation, Indianapolis, IN). Cell lysates were removed using a plastic cell scraper. Lysates were incubated on ice for 10 min and clarified, by centrifugation at 14,000 x g for 15 min at 4°C. The clarified lysates were assayed for protein content using a BCATM protein assay (Thermo Scientific, Rockford, IL). SDS-PAGE gel electrophoresis and western blotting were performed using standard techniques. A mouse monoclonal antibody to Cldn-2 (2 ug/ul) that detects a band of approximately 22-23 kDa was added in 5% skim milk at 4°C overnight. A secondary antibody, anti-Mouse (1:1000), in 5% skim milk was applied for 1 hour.  $\beta$ -actin was used as a loading control.

#### *Statistics Analyses*

Statistical analyses and graphical summaries were performed using GraphPad Prism ® version 6.00 for Windows (GraphPad Software Inc., La Jolla, CA). All values are presented as means  $\pm$  SEM. An unpaired t-test was used to analyze experiments conducted on two groups. When

three or more groups were studied an unpaired t-test were used to compare each experimental treatment to control of the same time point. For all statistical analyses, differences with a P value <0.05 were considered significant.

## **Results and Discussion**

### *Transport of phenol red in Caco-2 monolayers grown on collagen coated polytetrafluoroethylene Transwell supports*

We initially attempted to assess paracellular Ca flux in Caco-2 cells using phenol red. Caco-2 cells were treated either TB (control) or raffinose, which is known to non-specifically open the tight junction. Unexpectedly, treatment with raffinose for 90 minutes did not alter phenol red transport in Caco-2 cells. In fact, phenol red flux was suppressed in cells exposed to raffinose for 120 minutes ( $P = 0.006$ ; Figure 1a). To determine if the duration of exposure to raffinose was insufficient to measure an effect on paracellular transport, the treatment was extended to 4 hours. Exposing Caco-2 cells to raffinose for 4 hours resulted in a 1.2-fold increase in phenol red transport compared to TB (control;  $P = 0.17$ ; Figure 1b). The fact that phenol red was without a significant effect in this model prompted a reevaluation of all aspects of this in vitro system. Upon review it was noted that the collagen coated polytetrafluoroethylene Transwell supports used in this experiment have a membrane thickness of 50  $\mu\text{m}$  which is five-times the thickness of polyester Transwell permeable inserts. We hypothesized that the relatively thick membrane represented a physical barrier that precluded us from detecting a change in paracellular Ca transport. We therefore, substituted the polytetrafluoroethylene supports for polyester inserts, and replaced phenol red with  $^{45}\text{Ca}$ , a marker specific for calcium flux, in an effort to improve the sensitivity and specificity of the model.

#### *Validation of total transepithelial <sup>45</sup>Ca Transwell transport model*

Transwell inserts with a semipermeable membrane were previously used to examine transepithelial, transcellular and paracellular transport pathways in the presence of various drugs and nutrients. Such a model requires independent validation as a wide variety of methodologies have been previously published (23-25, 72, 76, 77, 79). As expected, treatment with 1,25-dihydroxyvitamin D significantly increased transepithelial Ca transport (Figure 2; 2-fold increase;  $P=0.0132$ ), indicating that this model responds normally to vitamin D. To further validate the model, the apical side of the Caco-2 monolayer was exposed to an acidic environment by the addition of a low pH buffer (pH = 3) to the upper chamber. Total transepithelial Ca flux was 4.5-fold higher after treatment with an acidic buffer compared to TB with a neutral pH (Figure 3). This observation is in agreement with in vitro work by Tang and Goodenough who demonstrated increased Ca permeability in a low pH environment (80).

#### *Acute effect of amino acids on total transepithelial <sup>45</sup>Ca transport*

Our primary objective was to assess if amino acids have a direct effect on the enterocyte and thereby increase calcium transport. To assess the effect of amino acids on total transepithelial calcium transport, we first supplemented TB with an amount of amino acids comparable to that found in high glucose DMEM (Table), which we considered to be moderate in amino acid concentration (MAA). High glucose DMEM is the primary constituent of regular Caco-2 growth media and contains more amino acids than other commercially available media such as EMEM. For this reason we did not consider the amount of amino acids provided by the MAA treatment to be low or restrictive. Alternatively, we did not feel that this treatment provided additional amino acid supplementation above that provided by normal growth media. For these experiments we exposed the apical side or top of the monolayer and both the top and bottom sides of the monolayer to MAA. Exposing both sides of the Caco-2 monolayer to MAA for 6 hours did not alter calcium transport compared to control (TB). However, the addition of MAA to

the top chamber resulted in a 1.6-fold increase in total transepithelial calcium transport (Figure 4;  $P=0.069$ ). As with most nutrient effects, the effect of MAA treatment appeared modest and was comparable to the previously reported trans-10, cis-12 conjugated linoleic acid-induced 2-fold increase in total transepithelial  $^{45}\text{Ca}$  transport (25). Our in vivo experiments in both rats and humans examined intestinal Ca transport after 5-7 days of dietary protein supplementation. We therefore, were interested in whether a longer in vitro treatment period in our Caco-2 cell model would have a greater effect on total transepithelial transport. However, with longer treatment protocols the issue of nutrient deprivation in cells cultured in low amino acid conditions needed to be addressed. To resolve these concerns, the TB control was reformulated.

*Effect of prolonged amino acid supplementation on total transepithelial  $^{45}\text{Ca}$  transport:*

*Preliminary Results*

Prolonged amino acid supplementation studies consisted of 72 hours of amino acid pretreatment followed by 6 hour assessment of total transepithelial Ca transport in the presence of glutamine, glycine and 12 additional EAA in TB (control) or HAA treatment (Table). The TB control used in these experiments differed from our original TB in that it contained glycine (0.03 mg/mL) at a concentration identical to that found in high glucose DMEM and EAA (1.2 mg/mL), and provided a final concentration of 1.778 mg total amino acids/mL. As in our previous experiments, both conditions contained L-glutamine (a source of nitrogen for the synthesis of vitamins, amino acids and nucleotides), which is necessary for cell proliferation and differentiation. In nutrient-deprived states L-glutamine can be used for energy and maintenance of protein synthesis in Caco-2 cells (81-83). Thus, these experiments were designed to examine the effect of amino acid supplementation beyond the amount provided by normal growth media, and represents a better in vitro model for our human and animal studies (38, 58) than the acute amino acid transport experiments (described above). Prolonged amino acid supplementation significantly augmented total transepithelial Ca transport compared to control (Figure 5; 1.6-fold



increase;  $P=0.0048$ ). These data are from one experiment and need to be repeated in order to confirm this potentially important finding.

To date, it is unclear if amino acids exert an effect on the transcellular, paracellular or both calcium absorption pathways as our model cannot distinguish between these two transport mechanisms. The microarray screen summarized in the Introduction raises the possibility that dietary protein or amino acids augment paracellular Ca transport by increasing expression of tight junction protein Cldn-2. Since Cldn-2 is a Ca specific, pore-forming Cldn, it is reasonable to believe that Cldn-2 has a major role in Ca transport. In order to directly address this hypothesis, Cldn-2 was stably suppressed in Caco-2 cells and  $^{45}\text{Ca}$  transport studies were repeated.

#### *Confirmation of Cldn-2 knockdown in Caco-2 Cells*

qPCR analysis using RNA isolated from Caco-2 cells expressing shRNAs directed against Cldn-2 demonstrated effective knockdown of Cldn-2, with two of a panel of 5 shRNAs resulting in a  $\geq 88\%$  suppression of the Cldn-2 transcript (Figure 6a-b). Western blot analysis revealed that shRNA 3 was the most effective shRNA (6c-d). Scrambled shRNA did not appear to alter the level of expression of Cldn-2 (6c-d), which suggests that lentiviral infection did not induce off-target effects. GFP expression, a marker of transduction efficiency and shRNA expression, appeared relatively consistent between Caco-2 cells infected with scrambled and Cldn-2 specific shRNAs (6e).

#### *Effect of stable Cldn-2 knockdown on total transepithelial $^{45}\text{Ca}$ transport*

Of the 23 distinct Cldns identified in humans (84), Cldn-2 and -12 are characterized as Ca permissive tight junction proteins, which, through transient knockdown in Caco-2 cells, were proposed to be involved in 1,25-dihydroxyvitamin D-induced Ca transport (7). Overexpression of Cldn-2 and Cldn-12, but not Cldn-7 increased Ca transport in Caco-2 cells (7).

As shown in Figure 7, stable knockdown of Cldn-2 in post-confluent Caco-2 cells elicited more than a 3-fold reduction in total transepithelial Ca transport ( $P < 0.0001$ ). To our knowledge we are the first to demonstrate an independent role for endogenous Cldn-2 in total transepithelial Ca transport in Caco-2 cells. Overexpression studies are inherently limited since they result in highly unphysiologic levels of the target protein. Our knockdown study establishes a role for Cldn-2 in Ca transport at endogenous levels in Caco-2 cells. It is not unreasonable to believe that Cldn-2 exhibits a functional role in intestinal Ca transport, as other members of the Cldn family are important mediators of Ca handling in other tissues. For example, under low dietary Ca conditions, Cldn-16 and -19 function together to form a Ca selective pore for reabsorption of Ca in the thick ascending limb of Henle. Conversely, when Ca intake is high, activation of the calcium sensing receptor results in upregulation of Cldn-14. Cldn-14, in turn, can interact with Cldn-16 to block Ca reabsorption and increase Ca excretion by the kidney (85). Results from the present study provide further support for Cldn regulation of the Ca economy.

As noted in the Introduction, we have initially focused on Cldn-2 based on the results of our microarray screen. Our Caco-2 cell line in which Cldn-2 was largely eliminated can now be used to examine the contribution of Cldn-2 to the effects of amino acids on total transepithelial Ca transport. It will also be of interest to investigate if diminished expression of Cldn-2 disrupts tight junction architecture or alters expression of other tight junction proteins such as cation pore sealers that could impede Ca permeability (4).

Despite its several strengths, our in vitro model also has limitations. Post-confluent Caco-2 cells provide a well-established in vitro model of the small intestine, however, this model does not allow for differentiation between segments of the small intestine. In the Caco-2 cell model, the pH of the culture media was fixed between 7.37-7.4. As shown in Figure 3, pH has a profound effect on Ca permeability. Since the pH varies along the course of the small intestine our model does not adequately capture the pH influence on Ca transport (86). Lastly, our current model

cannot distinguish between transcellular and paracellular Ca transport. However, with the use of dyes such as lucifer yellow which is restricted to the extracellular space should in combination with  $^{45}\text{Ca}$  transport studies allow us to assess the contribution of paracellular Ca transport to the effect of amino acids on transepithelial Ca transport.

## **Conclusion**

In summary, the current study established and validated an in vitro model for  $^{45}\text{Ca}$  transport. The model permitted an initial assessment of the direct effect of amino acids on total transepithelial Ca transport. A trend towards increased total transepithelial Ca transport was observed when the apical side of post-confluent Caco-2 cell monolayers was exposed to essential and non-essential amino acids for 6 hours. Preliminary results from prolonged amino acid treatment experiments showed significantly enhanced Ca transport, however, corroborative studies are warranted. In subsequent studies, the Caco-2 transport system was used to establish the role of endogenous Cldn-2 in total transepithelial Ca transport, and demonstrated a significant reduction in Ca transport when Cldn-2 was suppressed. This is the first study to generate a stable Cldn-2 knockdown in Caco-2 cells and provides evidence for role of Cldn-2 in Ca transport in the absence of pharmacologic or hormonal agonists. The present work will also allow us to directly address the contribution of Cldn-2 to amino acid-induced increases in Ca transport.

**Table.** Amino Acid Treatments

Amino Acid (mg/mL)	DMEM	MAA <sup>1</sup>	HAA <sup>1,2</sup>
Glycine	0.030	0.016	0.085
L-Arginine hydrochloride	0.084	0.263	0.922
L-Cystine 2HCl	0.063	0.050	0.175
L-glutamine	0.584	0.585	0.585
L-Histidine hydrochloride H2O	0.042	0.088	0.306
L-Isoleucine	0.105	0.109	0.382
L-Leucine	0.105	0.109	0.382
L-Lysine hydrochloride	0.146	0.151	0.529
L-Methionine	0.030	0.031	0.110
L-Phenylalanine	0.066	0.069	0.241
L-Serine	0.042	0.022	0.077
L-Threonine	0.095	0.099	0.347
L-Tryptophan	0.016	0.021	0.074
L-Tyrosine disodium salt dihydrate	0.104	0.075	0.263
L-Valine	0.094	0.098	0.341
L-Alanine		0.019	0.065
L-Asparagine		0.028	0.096
L-Aspartic Acid		0.028	0.097
L-Gutamic Acid		0.031	0.107
L-Proline		0.024	0.084
<b>Total Amino Acids (mg/mL)</b>	<b>1.606</b>	<b>1.914</b>	<b>5.267</b>

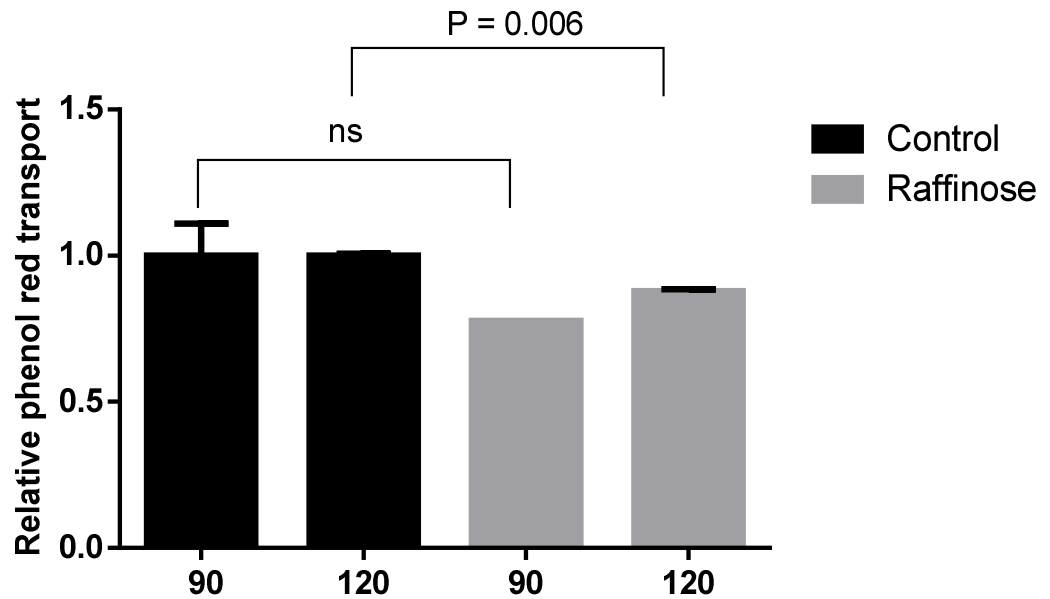
<sup>1</sup>Includes glutamine from transport buffer<sup>2</sup>Includes glycine from transport buffer

MAA, moderate amino acid transport treatment

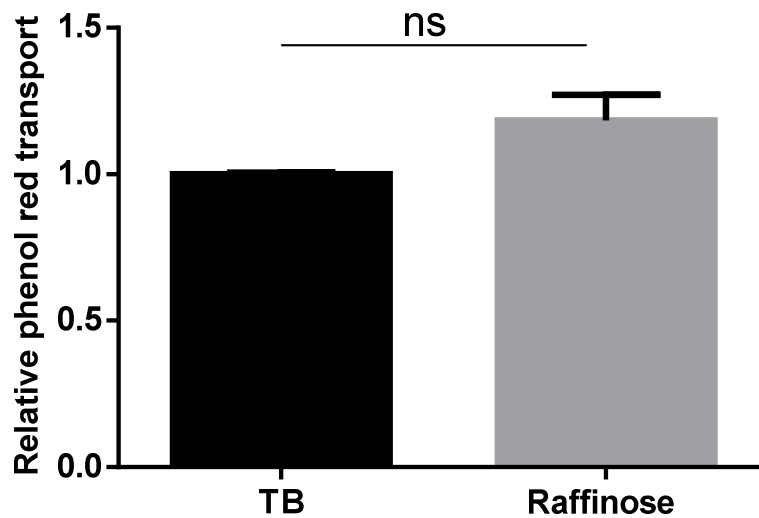
HAA, high amino acid transport treatment

## Figures

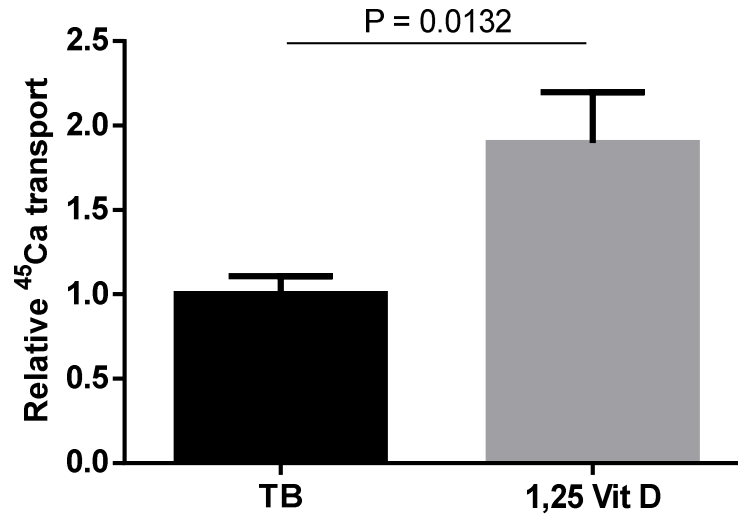
a



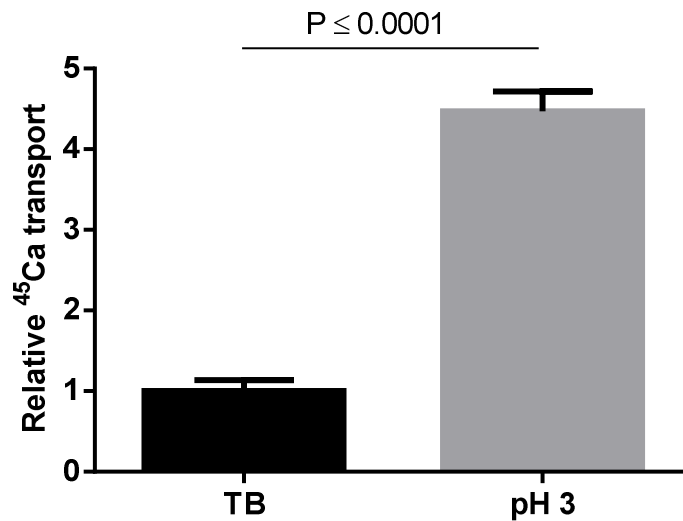
b



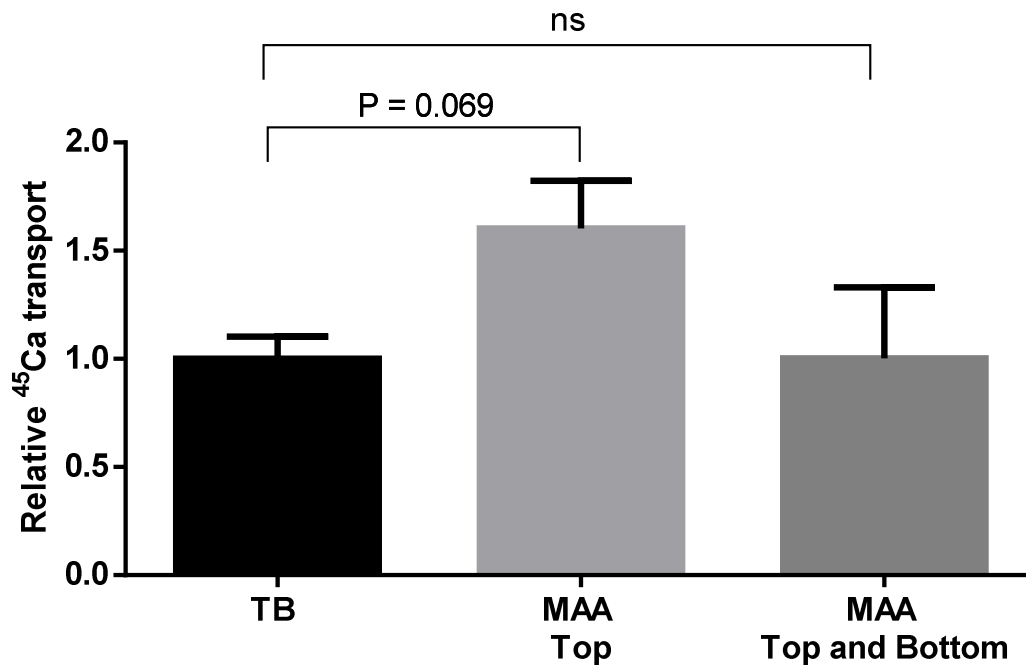
**Figure 1a-b.** Relative diffusional phenol red transport (top to bottom) across monolayers of Caco-2 cells (Mooseker, approximately 21-day post confluent) at 90 and 120 min (**1a**) and 4 hours (**1b**).  $n = 2$  monolayers grown on collagen coated polytetrafluoroethylene Transwell inserts exposed to transport buffer (TB, control) or 100 mM raffinose.



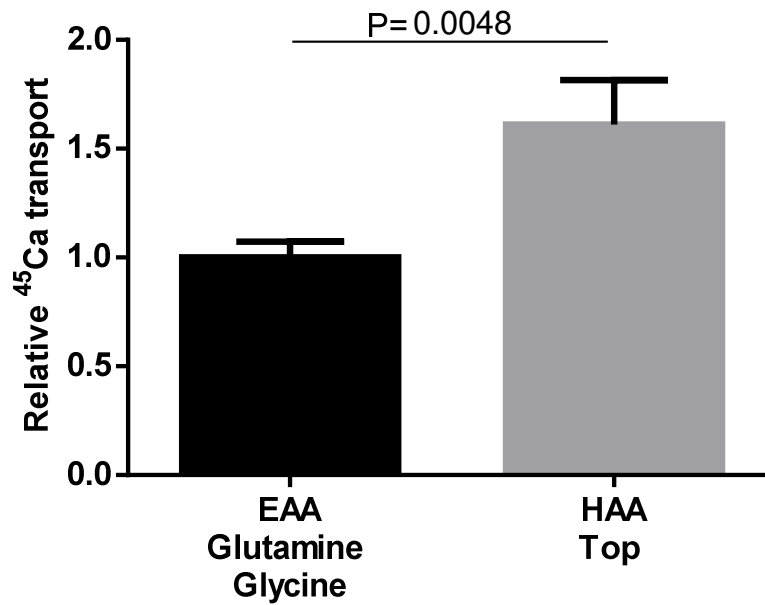
**Figure 2.** Total transepithelial <sup>45</sup>Ca transport across monolayers of Caco-2 cells (Mooseker, approximately 13-17 days post confluences) at 4 hours. Monolayers grown on polyester membrane Transwell inserts exposed to transport buffer (TB, control) for 4 hours or 1,25 vitamin D added to 5% FBS Caco-2 growth media for 24 hr prior to transport study and then added to TB 4 hr during transport study. n=9 monolayers per condition.



**Figure 3.** Total transepithelial <sup>45</sup>Ca transport across monolayers of Caco-2 cells (Mooseker, 26-27 days in culture) at 6 hours. Monolayers grown on polyester membrane Transwell inserts exposed to transport buffer, pH of 7.37 (TB, control, n= 4) or acidic buffer, pH of 3 (added to the top chamber only, n= 3).

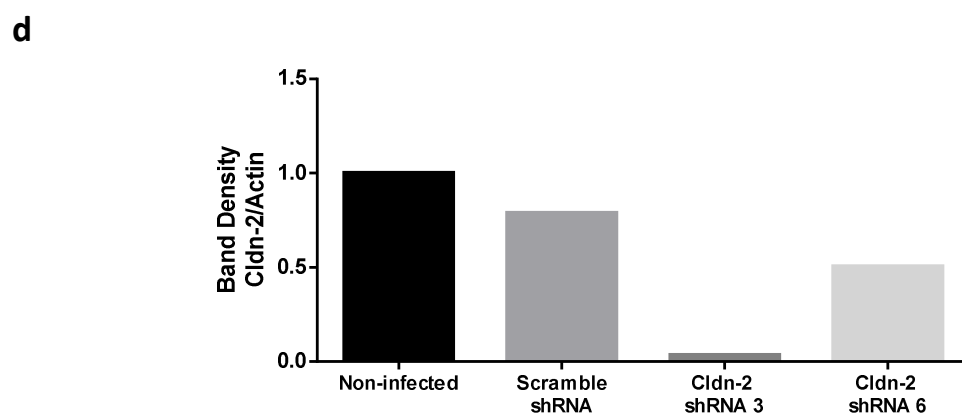
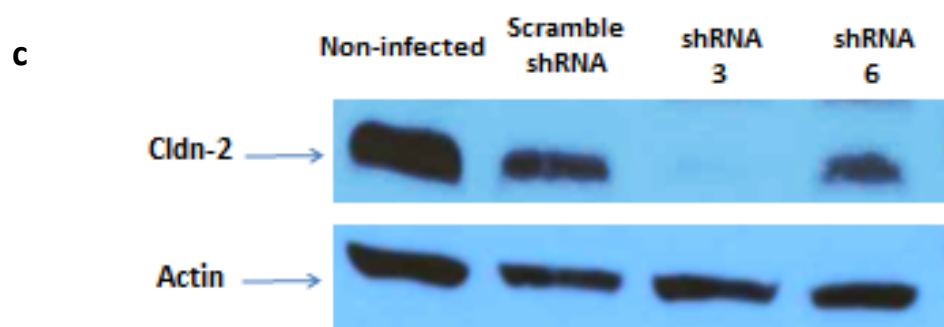
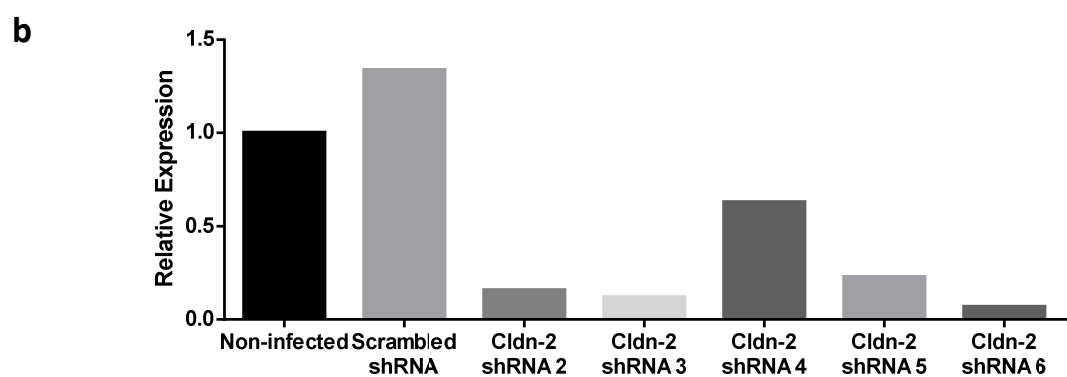
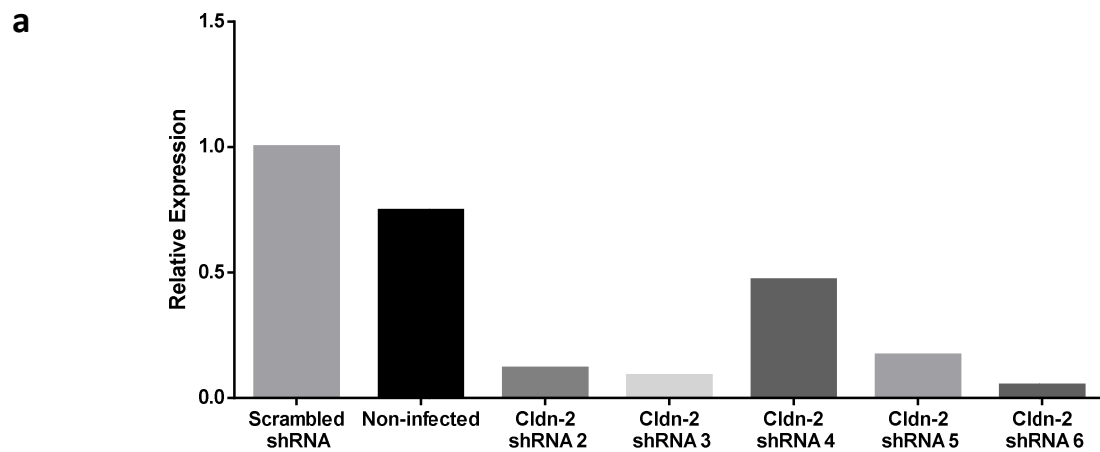


**Figure 4.** Total transepithelial  $^{45}\text{Ca}$  transport across monolayers of Caco-2 cells (Mooseker, 26 days in culture) at 6 hours. Monolayers grown on polyester membrane Transwell inserts exposed to transport buffer (TB, control), moderate amino acid treatment (MAA, amino acids added to TB) on one side of the monolayer (MAA Top), or MAA on both sides of the monolayer (MAA Top and Bottom).  $n=3$  monolayers per condition.

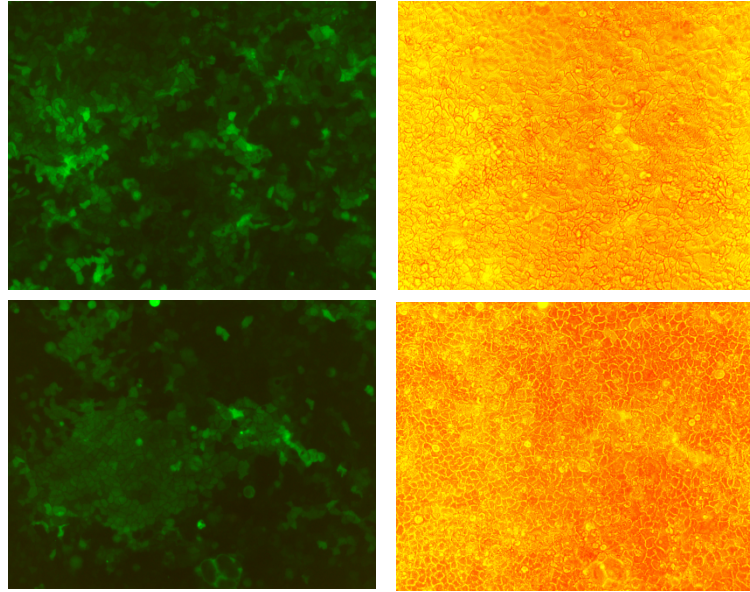


**Figure 5.** Monolayers grown on polyester membrane Transwell inserts (ATCC, 25 days in culture) pretreated with 1% FBS media (pretreatment control) or 1% FBS media supplemented with MAA (moderate amino acids) for 72 hours. Total transepithelial  $^{45}\text{Ca}$  transport at 6 hours in the presence of TB supplemented with glutamine, glycine and 12 additional essential amino acids (EAA) (control) or high amino acid treatment (HAA, amino acids added to control).

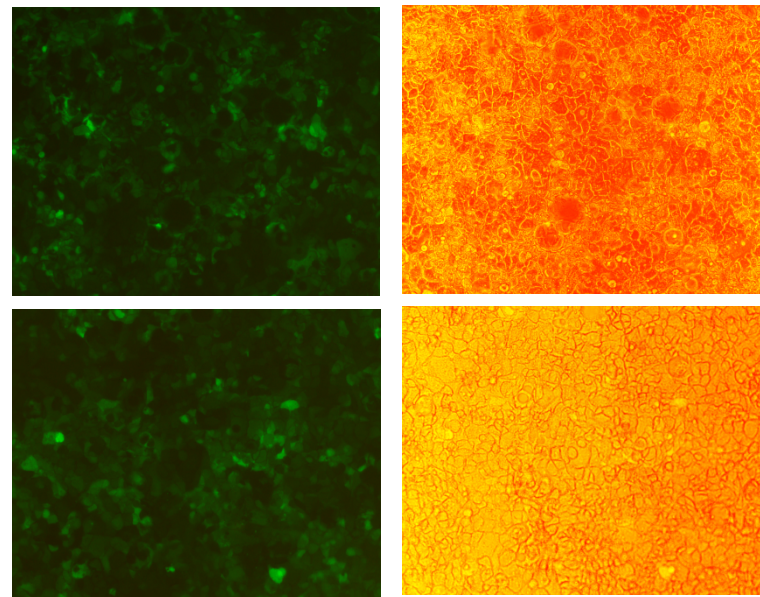




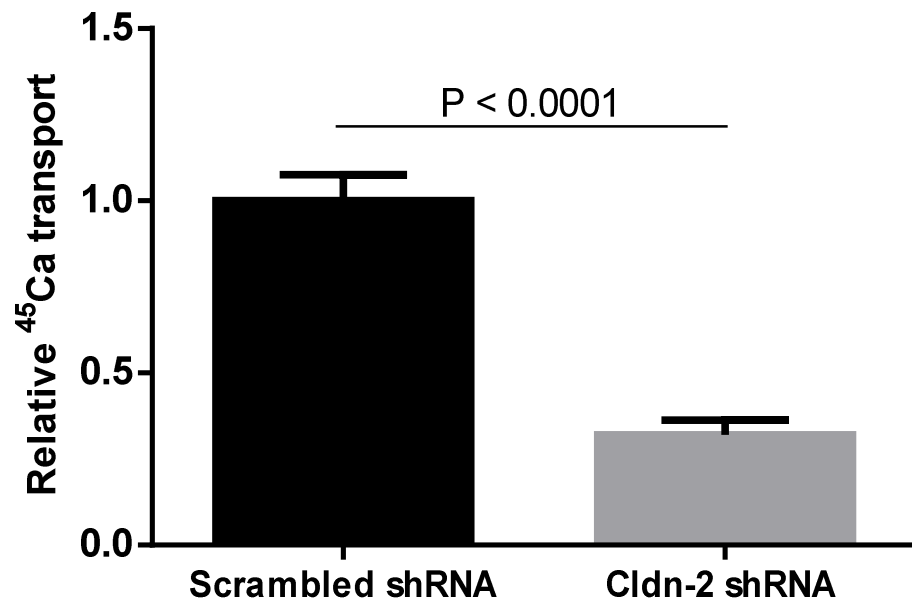
**e**



**f**



**Figure 6a-f.** Confirmed knockdown efficiency of claudin-2 (Cldn-2) in Caco-2 cells by lentiviral shRNA (Moosker, 2-weeks post confluence). Non-infected Caco-2 cells (control), Caco-2 cells infected with non-silencing, scrambled shRNA (control) or claudin-2 specific shRNAs. Confirmation of knockdown of Cldn-2 by qPCR (**a-b**, control: GAPDH). Confirmation of knockdown of Cldn-2 by Western blot ( $\beta$ -actin used as a loading control, **c**). Relative expression of Cldn-2 in **c** normalized to  $\beta$ -actin, **d**). Caco-2 cells infected with scrambled shRNA (control) stably expressing GFP (**e**, *left panels*: fluorescent light, *right panels*: white light) or claudin-2 specific shRNA 3 stably expressing GFP (**f**, *left panels*: fluorescent light, *right panels*: white light) imaged by fluorescent microscopy.



**Figure 7.** Total transepithelial  $^{45}\text{Ca}$  transport across monolayers of Caco-2 cells infected with scrambled (control) or claudin-2 specific lentiviral shRNA (Mooseker, 25 days in culture). Transport assessed at 6 hrs in transport buffer supplemented with glycine, glutamine and 12 additional essential amino acids (EAA). n=6 monolayers per condition.

## **Chapter 5:**

### **Conclusions and Future Study Directions**

We previously supplemented a low protein diet with dibasic amino acids and observed a trend towards increased Ca absorption in young women. Our sample size of 14 and six day study design may have hindered us from observing a statistically significant effect. Larger and longer clinical trials should provide more robust estimates of the magnitude of this effect and its duration. It would be of interest to explore the impact of dibasic amino acids along with other combinations of amino acids on calcium economy in populations who are at risk for calcium malabsorption. For example individuals with Crohn's or celiac disease would benefit from non-satiating, nutrient based, low-risk, nutritional therapies to optimize calcium absorption. Furthermore, individuals with protein intakes below the RDA may benefit from amino acid supplementation to improve Ca economy. Lastly, the effect of amino acid supplementation on Ca absorption may prove to be beneficial during critical stages in life such as infancy.

We have established and validated an in vitro model for  $^{45}\text{Ca}$  transport in Caco-2 cells and have preliminary evidence to suggest that 72 hours of treatment with amino acid enriched media increases total transepithelial Ca transport. Future experiments will need to corroborate these findings. Our current manual method requires an approximately 25-day culture period and allows for up to 24-wells per experiment. Recent advances in culturing systems have decreased the required culturing period for Caco-2 cell differentiation to 3-7 days (87), allowing for increased efficiency and decreased risk of contamination. Furthermore, by collaborating with pharmaceutical companies we can automate this process with the use of a liquid handler and articulated arm (86), thereby increasing standardization and reducing inter- and intra-assay variability brought forth by human error.

Our current in vitro Ca transport system can be used in the future to assess the effect of different combinations and concentrations of amino acids on total transepithelial transport. Understanding the minimal amino acid requirements of Caco-2 cells will allow us to further improve our control treatment. We may be able to lower the amino acid concentration of our control without causing nutrient deprivation or damage, which could increase the magnitude of effect observed with amino acid supplementation. Cell viability studies and assessment of lactase dehydrogenase release after exposure to different amino acid concentrations will be informative in evaluating any potential cytotoxic effects of our treatments. To better understand the contribution of paracellular Ca transport to the effect of amino acids on transepithelial transport, dyes such as lucifer yellow which is restricted to the extracellular space, can be included in our model.

Data from our laboratory provide evidence for the role of Cldn-2 in total transepithelial Ca transport in vitro. We recently generated a stable Cldn-2 knockdown cell line and observed a significant decline in total transepithelial Ca transport. We plan to use this model to address the contribution of Cldn-2 to amino acid-induced increases in Ca transport. In order to corroborate our in vitro findings, it is necessary to explore the role of Cldn-2 in Ca transport in vivo. This would require conditional deletion of Cldn-2 in a mouse model. For these experiment, Villin-cre transgenic mice (JAX® Mice database, B6.SJL-Tg(Vil-cre)997Gum/J), who express Cre recombinase under the direction of the mouse villin 1 promoter, would be crossed with a strain containing a loxP site-flanked sequence for Cldn-2. Cre-mediated recombination would allow for tissue-specific deletion of Cldn-2. Recombination would occur in villi and crypt cells of the small and large intestines, thereby generating gut-specific gene-deficient mice for Cldn-2. Ca absorption could be assessed by <sup>45</sup>Ca skeletal incorporation.

## References

1. Hoenderop JG, Nilius B, Bindels RJ. Calcium absorption across epithelia. *Physiol Rev*. 2005 Jan;85:373-422.
2. Bronner F, Pansu D. Nutritional aspects of calcium absorption. *J Nutr*. 1999 Jan;129:9-12.
3. Van Itallie CM, Anderson JM. Claudins and epithelial paracellular transport. *Annu Rev Physiol*. 2006;68:403-29.
4. Hu YJ, Wang YD, Tan FQ, Yang WX. Regulation of paracellular permeability: factors and mechanisms. *Molecular biology reports*. 2013 Nov;40:6123-42.
5. Krug SM, Gunzel D, Conrad MP, Lee IF, Amasheh S, Fromm M, Yu AS. Charge-selective claudin channels. *Annals of the New York Academy of Sciences*. 2012 Jun;1257:20-8.
6. Amasheh S, Meiri N, Gitter AH, Schoneberg T, Mankertz J, Schulzke JD, Fromm M. Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. *J Cell Sci*. 2002 Dec 15;115:4969-76.
7. Fujita H, Sugimoto K, Inatomi S, Maeda T, Osanai M, Uchiyama Y, Yamamoto Y, Wada T, Kojima T, et al. Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent  $\text{Ca}^{2+}$  absorption between enterocytes. *Mol Biol Cell*. 2008 May;19:1912-21.
8. Coward KH, Kassner EW, Waller LW. Availability of the Calcium of Milk. *British medical journal*. 1943 Jul 10;2:39-40.
9. Vaughan OW, Filer LJ, Jr. The enhancing action of certain carbohydrates on the intestinal absorption of calcium in the rat. *J Nutr*. 1960 May;71:10-4.
10. Fournier P, Dupuis Y, Susbielle H, Allez M, Tardy N. [Mode of action of building glucides on the utilization of calcium]. *Journal de physiologie*. 1955;47:351-62.
11. Lengemann FW. The site of action of lactose in the enhancement of calcium utilization. *J Nutr*. 1959 Sep;69:23-7.

12. Wasserman RH, Comar CL, Schooley JC, Lengemann FW. Interrelated effects of L-lysine and other dietary factors on the gastrointestinal absorption of calcium <sup>45</sup> in the rat and chick. *J Nutr.* 1957 Jul 10;62:367-76.
13. Civitelli R, Villareal DT, Agnusdei D, Nardi P, Avioli LV, Gennari C. Dietary L-lysine and calcium metabolism in humans. *Nutrition.* 1992 Nov-Dec;8:400-5.
14. Leichter J, Tolensky AF. Effect of dietary lactose on the absorption of protein, fat and calcium in the postweaning rat. *Am J Clin Nutr.* 1975 Mar;28:238-41.
15. Armbrecht HJ, Wasserman RH. Enhancement of Ca<sup>++</sup> uptake by lactose in the rat small intestine. *J Nutr.* 1976 Sep;106:1265-71.
16. Schuette SA, Knowles JB, Ford HE. Effect of lactose or its component sugars on jejunal calcium absorption in adult man. *Am J Clin Nutr.* 1989 Nov;50:1084-7.
17. Zittermann A, Bock P, Drummer C, Scheld K, Heer M, Stehle P. Lactose does not enhance calcium bioavailability in lactose-tolerant, healthy adults. *Am J Clin Nutr.* 2000 Apr;71:931-6.
18. DeGrazia JA, Ivanovich P, Fellows H, Rich C. A double isotope method for measurement of intestinal absorption of calcium in man. *The Journal of laboratory and clinical medicine.* 1965 Nov;66:822-9.
19. Lee MF, Krasinski SD. Human adult-onset lactase decline: an update. *Nutrition reviews.* 1998 Jan;56:1-8.
20. Montgomery RK, Krasinski SD, Hirschhorn JN, Grand RJ. Lactose and lactase--who is lactose intolerant and why? *Journal of pediatric gastroenterology and nutrition.* 2007 Dec;45 Suppl 2:S131-7.
21. Stathos TH, Shulman RJ, Schanler RJ, Abrams SA. Effect of carbohydrates on calcium absorption in premature infants. *Pediatric research.* 1996 Apr;39:666-70.
22. Abrams SA, Griffin IJ, Davila PM. Calcium and zinc absorption from lactose-containing and lactose-free infant formulas. *Am J Clin Nutr.* 2002 Aug;76:442-6.

23. Jewell C, Cashman KD. The effect of conjugated linoleic acid and medium-chain fatty acids on transepithelial calcium transport in human intestinal-like Caco-2 cells. *Br J Nutr.* 2003 May;89:639-47.
24. Jewell C, Cusack S, Cashman KD. The effect of conjugated linoleic acid on transepithelial calcium transport and mediators of paracellular permeability in human intestinal-like Caco-2 cells. *Prostaglandins Leukot Essent Fatty Acids.* 2005 Mar;72:163-71.
25. Murphy EF, Hooiveld GJ, Muller M, Calogero RA, Cashman KD. Conjugated linoleic acid alters global gene expression in human intestinal-like Caco-2 cells in an isomer-specific manner. *J Nutr.* 2007 Nov;137:2359-65.
26. Van Beers EH, Al RH, Rings EH, Einerhand AW, Dekker J, Buller HA. Lactase and sucrase-isomaltase gene expression during Caco-2 cell differentiation. *The Biochemical journal.* 1995 Jun 15;308 ( Pt 3):769-75.
27. Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stamatii A, Zucco F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell biology and toxicology.* 2005 Jan;21:1-26.
28. Findley MK, Koval M. Regulation and roles for claudin-family tight junction proteins. *IUBMB Life.* 2009 Apr;61:431-7.
29. Rahner C, Mitic LL, Anderson JM. Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. *Gastroenterology.* 2001 Feb;120:411-22.
30. Kiuchi-Saishin Y, Gotoh S, Furuse M, Takasuga A, Tano Y, Tsukita S. Differential expression patterns of claudins, tight junction membrane proteins, in mouse nephron segments. *Journal of the American Society of Nephrology : JASN.* 2002 Apr;13:875-86.
31. Van Itallie C, Rahner C, Anderson JM. Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. *J Clin Invest.* 2001 May;107:1319-27.



32. Hou J, Renigunta A, Yang J, Waldegger S. Claudin-4 forms paracellular chloride channel in the kidney and requires claudin-8 for tight junction localization. *Proc Natl Acad Sci U S A*. 2010 Oct 19;107:18010-5.
33. Shapses SA, Sukumar D, Schneider SH, Schlussek Y, Brolin RE, Taich L. Hormonal and dietary influences on true fractional calcium absorption in women: role of obesity. *Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA*. 2012 Nov;23:2607-14.
34. Ramsbeck K, Keuler NS, Davis LA, Hansen KE. Factors Associated with Calcium Absorption in Postmenopausal Women: A Post Hoc Analysis of Dual-Isotope Studies. *Journal of the Academy of Nutrition and Dietetics*. 2013 Oct 24.
35. Hunt JR, Johnson LK, Fariba Roudhead ZK. Dietary protein and calcium interact to influence calcium retention: a controlled feeding study. *Am J Clin Nutr*. 2009 May;89:1357-65.
36. Ceglia L, Harris SS, Abrams SA, Rasmussen HM, Dallal GE, Dawson-Hughes B. Potassium bicarbonate attenuates the urinary nitrogen excretion that accompanies an increase in dietary protein and may promote calcium absorption. *J Clin Endocrinol Metab*. 2009 Feb;94:645-53.
37. Kerstetter JE, O'Brien KO, Insogna KL. Dietary protein affects intestinal calcium absorption. *Am J Clin Nutr*. 1998 Oct;68:859-65.
38. Kerstetter JE, O'Brien KO, Caseria DM, Wall DE, Insogna KL. The impact of dietary protein on calcium absorption and kinetic measures of bone turnover in women. *J Clin Endocrinol Metab*. 2005 Jan;90:26-31.
39. Lee YS, Noguchi T, Naito H. Phosphopeptides and soluble calcium in the small intestine of rats given a casein diet. *The British journal of nutrition*. 1980 May;43:457-67.
40. Mykkanen HM, Wasserman RH. Enhanced absorption of calcium by casein phosphopeptides in rachitic and normal chicks. *The Journal of nutrition*. 1980 Nov;110:2141-8.

41. Tsuchita H, Sekiguchi I, Kuwata T, Igarashi C, Ezawa I. The effect of casein phosphopeptides on calcium utilization in young ovariectomized rats. *Zeitschrift fur Ernahrungswissenschaft*. 1993 Jun;32:121-30.
42. R Brommage MJ, R Jost. Influence of casein phosphopeptides and lactulose on intestinal calcium absorption in adult female rats. *Lait*. 1991;71:173-80.
43. Narva M, Karkkainen M, Poussa T, Lamberg-Allardt C, Korpela R. Caseinphosphopeptides in milk and fermented milk do not affect calcium metabolism acutely in postmenopausal women. *Journal of the American College of Nutrition*. 2003 Feb;22:88-93.
44. Teucher B, Majsak-Newman G, Dainty JR, McDonagh D, FitzGerald RJ, Fairweather-Tait SJ. Calcium absorption is not increased by caseinophosphopeptides. *The American journal of clinical nutrition*. 2006 Jul;84:162-6.
45. Sherman. Calcium requirement of maintenance in man. *J Biol Chem*. 1920;44:21-7.
46. Kerstetter JE, O'Brien KO, Insogna KL. Low protein intake: the impact on calcium and bone homeostasis in humans. *J Nutr*. 2003 Mar;133:855S-61S.
47. Bushinsky DA. Acid-base imbalance and the skeleton. *Eur J Nutr*. 2001 Oct;40:238-44.
48. Remer T. Influence of diet on acid-base balance. *Semin Dial*. 2000 Jul-Aug;13:221-6.
49. Barzel US, Massey LK. Excess dietary protein can adversely affect bone. *J Nutr*. 1998 Jun;128:1051-3.
50. Bushinsky DA, Frick KK. The effects of acid on bone. *Current opinion in nephrology and hypertension*. 2000 Jul;9:369-79.
51. Anand CR, Linkswiler HM. Effect of protein intake on calcium balance of young men given 500 mg calcium daily. *J Nutr*. 1974 Jun;104:695-700.
52. Kim Y, Linkswiler HM. Effect of level of protein intake on calcium metabolism and on parathyroid and renal function in the adult human male. *J Nutr*. 1979 Aug;109:1399-404.
53. Allen LH, Oddoye EA, Margen S. Protein-induced hypercalciuria: a longer term study. *Am J Clin Nutr*. 1979 Apr;32:741-9.

54. Hegsted M, Linkswiler HM. Long-term effects of level of protein intake on calcium metabolism in young adult women. *J Nutr.* 1981 Feb;111:244-51.
55. Schuette SA, Hegsted M, Zemel MB, Linkswiler HM. Renal acid, urinary cyclic AMP, and hydroxyproline excretion as affected by level of protein, sulfur amino acid, and phosphorus intake. *J Nutr.* 1981 Dec;111:2106-16.
56. Schuette SA, Zemel MB, Linkswiler HM. Studies on the mechanism of protein-induced hypercalciuria in older men and women. *J Nutr.* 1980 Feb;110:305-15.
57. Spencer H, Kramer L, DeBartolo M, Norris C, Osis D. Further studies of the effect of a high protein diet as meat on calcium metabolism. *Am J Clin Nutr.* 1983 Jun;37:924-9.
58. Gaffney-Stomberg E, Sun BH, Cucchi CE, Simpson CA, Gundberg C, Kerstetter JE, Insogna KL. The effect of dietary protein on intestinal calcium absorption in rats. *Endocrinology.* Mar;151:1071-8.
59. T. Mitsuma NR, M. Kayama, Y. Mori, K. Adachi, Y. Yokoi, Jing Ping, T. Nogimori, Y. Hirooka. Distribution of calcium sensing receptor in rats: an immunohistochemical study. *Endocrine Regulations.* 1999;33:55-9.
60. Conigrave AD, Quinn SJ, Brown EM. L-amino acid sensing by the extracellular Ca<sup>2+</sup>-sensing receptor. *Proc Natl Acad Sci U S A.* 2000 Apr 25;97:4814-9.
61. Conigrave AD, Franks AH, Brown EM, Quinn SJ. L-amino acid sensing by the calcium-sensing receptor: a general mechanism for coupling protein and calcium metabolism? *Eur J Clin Nutr.* 2002 Nov;56:1072-80.
62. Busque SM, Kerstetter JE, Geibel JP, Insogna K. L-type amino acids stimulate gastric acid secretion by activation of the calcium-sensing receptor in parietal cells. *Am J Physiol Gastrointest Liver Physiol.* 2005 Oct;289:G664-9.
63. Dawson-Hughes B, Harris SS, Rasmussen HM, Dallal GE. Comparative effects of oral aromatic and branched-chain amino acids on urine calcium excretion in humans. *Osteoporos Int.* 2007 Jul;18:955-61.

64. Pothiwala P, Evans EM, Chapman-Novakofski KM. Ethnic variation in risk for osteoporosis among women: a review of biological and behavioral factors. *Journal of women's health*. 2006 Jul-Aug;15:709-19.
65. Kerstetter JE, Caseria DM, Mitnick ME, Ellison AF, Gay LF, Liskov TA, Carpenter TO, Insogna KL. Increased circulating concentrations of parathyroid hormone in healthy, young women consuming a protein-restricted diet. *Am J Clin Nutr*. 1997 Nov;66:1188-96.
66. BJ B. Synthesizing standardized mean-change measures. *British Journal of Mathematical and Statistical Psychology*. 1988;41:257-78.
67. Malabanan A, Veronikis IE, Holick MF. Redefining vitamin D insufficiency. *Lancet*. 1998 Mar 14;351:805-6.
68. Cohen J. *Statistical power analysis for the behavioral sciences*. 2nd ed. Hillsdale, NJ: Lawrence Earlbaum Associates; 1988.
69. Comar CL, Nold MM, Wasserman RH. The influence of amino acids and other organic compounds on the gastrointestinal absorption of calcium 45 and strontium 89 in the rat. *J Nutr*. 1956 Jul 10;59:371-83.
70. Mangano KM, Walsh SJ, Insogna KL, Kenny AM, Kerstetter JE. Calcium intake in the United States from dietary and supplemental sources across adult age groups: new estimates from the National Health and Nutrition Examination Survey 2003-2006. *J Am Diet Assoc*. May;111:687-95.
71. Austin GL, Ogden LG, Hill JO. Trends in carbohydrate, fat, and protein intakes and association with energy intake in normal-weight, overweight, and obese individuals: 1971-2006. *Am J Clin Nutr*. 2011 Apr;93:836-43.
72. Yee S. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man--fact or myth. *Pharmaceutical research*. 1997 Jun;14:763-6.

73. Balimane PV, Chong S, Morrison RA. Current methodologies used for evaluation of intestinal permeability and absorption. *Journal of pharmacological and toxicological methods*. 2000 Jul-Aug;44:301-12.
74. Chantret I, Rodolosse A, Barbat A, Dussaulx E, Brot-Laroche E, Zweibaum A, Rousset M. Differential expression of sucrase-isomaltase in clones isolated from early and late passages of the cell line Caco-2: evidence for glucose-dependent negative regulation. *J Cell Sci*. 1994 Jan;107 ( Pt 1):213-25.
75. Fleet JC, Eksir F, Hance KW, Wood RJ. Vitamin D-inducible calcium transport and gene expression in three Caco-2 cell lines. *Am J Physiol Gastrointest Liver Physiol*. 2002 Sep;283:G618-25.
76. Giuliano AR, Wood RJ. Vitamin D-regulated calcium transport in Caco-2 cells: unique in vitro model. *Am J Physiol*. 1991 Feb;260:G207-12.
77. Fleet JC, Wood RJ. Identification of calbindin D-9k mRNA and its regulation by 1,25-dihydroxyvitamin D3 in Caco-2 cells. *Arch Biochem Biophys*. 1994 Jan;308:171-4.
78. Suzuki T, Hara H. Various nondigestible saccharides open a paracellular calcium transport pathway with the induction of intracellular calcium signaling in human intestinal Caco-2 cells. *J Nutr*. 2004 Aug;134:1935-41.
79. Smetanova L, Stetinova V, Svoboda Z, Kvetina J. Caco-2 cells, biopharmaceutics classification system (BCS) and biowaiver. *Acta Medica (Hradec Kralove)*.54:3-8.
80. Tang VW, Goodenough DA. Paracellular ion channel at the tight junction. *Biophys J*. 2003 Mar;84:1660-73.
81. Pasiaka AE, Morgan JF. Glutamine metabolism of normal and malignant cells cultivated in synthetic media. *Nature*. 1959 Apr 25;183:1201-2.
82. Le Bacquer O, Laboisie C, Darmaun D. Glutamine preserves protein synthesis and paracellular permeability in Caco-2 cells submitted to "luminal fasting". *Am J Physiol Gastrointest Liver Physiol*. 2003 Jul;285:G128-36.

83. Murnin M, Kumar A, Li GD, Brown M, Sumpio BE, Basson MD. Effects of glutamine isomers on human (Caco-2) intestinal epithelial proliferation, strain-responsiveness, and differentiation. *Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract*. 2000 Jul-Aug;4:435-42.
84. Lal-Nag M, Morin PJ. The claudins. *Genome Biol*. 2009;10:235.
85. Gong Y, Renigunta V, Himmerkus N, Zhang J, Renigunta A, Bleich M, Hou J. Claudin-14 regulates renal Ca(+) transport in response to CaSR signalling via a novel microRNA pathway. *EMBO J*. 2012 Apr 18;31:1999-2012.
86. Balimane PV, Chong S. Cell culture-based models for intestinal permeability: a critique. *Drug discovery today*. 2005 Mar 1;10:335-43.
87. Yamashita S, Konishi K, Yamazaki Y, Taki Y, Sakane T, Sezaki H, Furuyama Y. New and better protocols for a short-term Caco-2 cell culture system. *Journal of pharmaceutical sciences*. 2002 Mar;91:669-79.